THE ROLE OF BONE MORPHOGENETIC PROTEIN 2 IN OVARIAN CANCER MIGRATION

by

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Abstract

The transforming growth factor-β (TGF-β) superfamily member bone morphogenetic protein 2 (BMP2) is over-expressed in epithelial ovarian cancer cells, and correlates with decreased survival time in advanced stage ovarian cancer patients. BMP2 has been shown to stimulate cell motility in ovarian cancer cell lines, however little is known about the molecular mechanisms underlying these pro-migratory effects. The aim of my study was to test the hypothesis that BMP2-induced ovarian cancer cell migration is mediated by the differential expression of cadherin cell adhesion molecules via activation of SMAD-dependent signaling.

Treatment of SKOV3 clear cell or endometrioid human ovarian cancer cells with BMP2 increased cell migration and N-cadherin mRNA and protein levels, while decreasing E-cadherin mRNA and protein levels. Importantly, small interfering RNA (siRNA)-mediated knockdown of N-cadherin inhibited BMP2-induced cell migration. BMP2 treatment induced both canonical SMAD1/5/8 phosphorylation and non-canonical SMAD2/3 phosphorylation, mediated by the BMP type I receptor ALK3, but not ALK2 or ALK6. Co-treatment with the BMP type I receptor inhibitors Dorsomorphin or DMH-1, but not the TGF-β type I receptor inhibitor SB-431542, reversed the effects of BMP2 on SMAD2/3 phosphorylation, N-cadherin (not blocked by SB-431542), and cell migration, but not E-cadherin. Moreover, BMP2-induced cell migration was reduced by siRNA-mediated knockdown of either SMAD2 or SMAD3. This study provides evidence of the possible dominant and critical role of N-cadherin over E-cadherin in promoting cancer cell migration.

Conversely, treatment of OVCAR8 high-grade serous human ovarian cancer cells with BMP2 did not increase cell migration and instead suppressed N-cadherin protein levels. BMP2

treatment in OVCAR8 also induced canonical SMAD1/5/8 phosphorylation, but not non-canonical SMAD2/3 phosphorylation.

Likewise, treatment of OVCAR5 high-grade serous human ovarian cancer cells with BMP2 yielded a marginal increase in cell migration while N-cadherin remains undetectable. BMP2 treatment in OVCAR5 also induced canonical SMAD1/5/8 phosphorylation and maybe non-canonical SMAD3 but not SMAD2 phosphorylation.

This study provides important insights into the molecular mechanisms underlying BMP2-induced human ovarian cancer cell migration. I report here that BMP2 induces SKOV3 ovarian cancer cell migration by up-regulating N-cadherin expression in a SMAD2/3-dependent manner, and that variability in the BMP2 response may be subtype-dependent.

Preface

This thesis is original, unpublished, independent work by the author, K. Lau.

This study was approved by the Children's and Women's Research Ethics Board (H98-70175-Ovarian cancer-CIHR).

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List of Abbreviations

ACVR2A	Activin receptor type-2A
ACVR2B	Activin receptor type-2B
ALK	Activin receptor-like kinase
AMH	Anti-Müllerian hormone
AMHR2	Anti-Müllerian hormone receptor, type II
ANOVA	Analysis of variance
BMPR2	Bone morphogenetic protein receptor type II
BMPs	Bone morphogenetic proteins
co-SMAD	Common SMAD
EMT	Epithelial-mesenchymal transition
ERK	Extracellular signal-regulated kinase
GDF	Growth differentiation factor
GDNF	Glial-derived neurotrophic factor
JNK	c-Jun NH2-terminal kinase
MAPK	Mitogen-activated protein kinase
MIS	Müllerian inhibiting substance
PI3K	Phosphatidylinositol 3-kinase
R-SMADs	Receptor-regulated SMADs
RT-qPCR	Reverse transcription quantitative real-time PCR
TGF-β	Transforming growth factor-β
TGFBR2	Transforming growth factor, beta receptor II

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Chapter 1: Introduction

1.1 Ovarian cancer

Ovarian cancer is the fifth leading cause of cancer death in women, and is the most lethal gynaecological cancer, despite accounting for less than 3% of new cancer cases in the United States [1]. This high lethality can be attributed to the fact that most diagnoses occur at a late stage where the five-year survival rate is approximately 30%, and is compounded by the lack of an effective screening strategy and subtle non-specific symptoms (reviewed in [2-4]). It follows that despite advancements in patient prognosis in other cancer types and a better understanding of ovarian cancer at the cellular and molecular biology level, the survival rate of women diagnosed with ovarian cancer has been little changed over the past 20 years (reviewed in [1, 5]). It is therefore important to gain a better understanding of the molecular mechanisms that promote ovarian cancer progression, which may have a major impact in the development of new therapeutics and improved screening strategies.

Over 90% of ovarian cancers are epithelial in origin, and can be classified into five major histological subtypes comprising of high-grade serous, clear cell, endometrioid, mucinous, and low-grade serous, from most to least common, respectively (reviewed in [6, 7]).

High-grade serous carcinoma is the most common subtype, accounting for approximately 70% of all ovarian carcinomas, with the majority of patients diagnosed at an advanced stage (III/IV) [8]. High-grade serous carcinomas present with a mixture of solid, glandular and transitional-like, and papillary growth patterns, and is frequently characterized by *TP*53 mutations (>95%), *BRCA1/2* mutations (30-45%), and high chromosomal instability (reviewed in [9]). Most (70-80%) high-grade serous carcinomas show good initial responsiveness to

platinum/taxane chemotherapy, although most tumours recur as a chemoresistant disease (reviewed in [10]).

Clear cell carcinomas are frequently associated with endometriosis, and accounts for approximately 10% of all ovarian carcinomas, with the majority of patients diagnosed at a low-stage (I/II) [8]. Clear cell carcinomas are frequently characterized by mutations in *ARID1A* (~50%) and *PIK3CA* (~40%), and up-regulation of mRNA and protein levels of HNF-1β (~100%) (reviewed in [9]).

Endometrioid carcinomas have a strong association with endometriosis, and similar to clear cell carcinomas, accounts for approximately 10% of all ovarian carcinomas, with the majority of patients diagnosed at a low-stage [11]. Endometrioid carcinomas are frequently characterized by mutations in *CTNNB1* (35-50%), *ARID1A* (~30%), and *PTEN* (~20%) (reviewed in [7, 9]).

Mucinous carcinomas account for less than 5% of all ovarian carcinomas and are typically found confined to one ovary, with the majority of tumours being borderline tumours or stage I [8]. Mucinous carcinomas are frequently characterized by *KRAS* mutations (>75%) and *HER2* amplification and overexpression (~20%) (reviewed in [9]).

Low-grade serous carcinomas, unlike high-grade serous carcinomas, are less common, accounting for approximately 3% of all ovarian carcinomas [8]. Low-grade serous carcinomas are frequently characterized by *KRAS* or *BRAF* mutations (60-70%) and are gnomically stable (reviewed in [9]).

The different histotypes of ovarian cancer are distinct diseases and should be approached as such in research and clinical settings.

1.1.1 Epithelial-mesenchymal transition

Epithelial-mesenchymal transition (EMT) has been described as the collection of molecular and morphological changes where epithelial cells transform from their compact organization in colonies into a mesenchymal state, adopting a spindle-shaped morphology with increased motility and invasiveness (reviewed in [12, 13]). The down-regulation of E-cadherin and up-regulation of N-cadherin is thought to be involved in EMT (reviewed in [12, 13]).

1.1.1.1 Proteins associated with EMT: cadherins

E- and N-cadherin are members of the cadherin superfamily that are transmembrane glycoproteins involved in cell-cell adhesion via Ca^{2+}-dependent interactions (reviewed in [14]). Additionally, reduction of E-cadherin expression has been correlated with invasiveness and metastasis in many epithelial cancers, and as a result, correlated with poor clinical prognosis (reviewed in [15]).

Primary ovarian tumours were found to express E-cadherin mRNA [16], which corresponds to immunohistochemical staining in the plasma membranes of serous, endometrioid, and mucinous tumours [17-19]. Interestingly, metastatic and ascites samples exhibited lower levels of E-cadherin relative to their originating solid ovarian tumour [16, 17, 19], and patients with negative E-cadherin staining exhibited a reduction in survival times [20, 21]. *In vitro* studies have demonstrated that the loss of E-cadherin-mediated cell-cell adhesion resulted in a significant increase in invasiveness of epithelial tumour cells [22-24], further supporting the tumour suppressive role of E-cadherin.

N-cadherin is commonly thought to serve pro-migratory and pro-invasive roles in cancer (reviewed in [25]), although the molecular basis of these roles is poorly understood. N-cadherin

was detected via immunohistochemical staining in the plasma membranes of serous and endometrioid ovarian tumours, but showed little to no staining in mucinous and clear cell ovarian tumours [18, 26-28]. In contrast to E-cadherin expression patterns, a significant inverse correlation between N-cadherin expression and survival was found among patients with high-grade serous ovarian cancer [29], further supporting the tumour promotor role of N-cadherin.

1.1.1.2 Transcriptional regulation of EMT

The transcription factor Snail represses the activity of the *E-cadherin* promoter via E-box binding [30, 31]. Snail mRNA was detected in primary ovarian tumours and metastatic material and found to be inversely correlated with E-cadherin mRNA levels [30, 32], although this correlation was not found at the protein level via immunoblotting or immunohistochemical staining [21, 32], which may be attributed to the unstable nature of the Snail protein [33]. Positive immunohistochemical staining of Snail in metastatic ovarian tumours, but not their corresponding originating tumours, was found to be associated with reduced overall survival [21].

Similar to Snail and belonging to the same family, the transcription factor Slug represses the activity of the *E-cadherin* promoter via E-box binding, which has been described in many tumours and cell lines [32, 34-37]. Slug mRNA was detected in ovarian cancer tumours and metastatic samples and found to be inversely correlated with E-cadherin mRNA levels [32]. Slug protein and mRNA levels were found to increase stepwise from benign to borderline, and from borderline to malignant ovarian tumours [38]. In contrast with Snail, Slug mRNA and protein levels were found to be correlated [32].

TWIST1 was found to repress the activity of the *E-cadherin* promoter in a similar manner to Snail and Slug [39], while binding to and inducing activity of the *N-cadherin* promoter via E-box binding [40]. TWIST1 was detected via immunohistochemical staining and immunoblotting of primary ovarian cancer specimens, whereby positive expression significantly predicted poorer patient outcomes relative to negative expression [41, 42]. *In vitro* studies support the tumour promoter role of TWIST1, as knockdown of TWIST1 with siRNA was shown to reduce cell motility and invasiveness in ovarian cancer cell lines [42].

ZEB1 was found to repress the activity of the *E-cadherin* promoter via E-box binding [43]. Conversely, shRNA-mediated knockdown of ZEB1 *in vitro* resulted in an increase of E-cadherin mRNA levels while reducing ovarian cancer cell motility and invasiveness [44]. High levels of ZEB1 mRNA was detected in ovarian cancer specimens [45], while protein levels were found to be elevated in high-grade serous ovarian cancer patients [46].

ZEB2 was found to repress the activity of the *E-cadherin* promoter via E-box binding [47], and has been implicated in promoting N-cadherin mRNA and protein expression in several epithelial cell lines [48, 49]. Elevated mRNA levels of ZEB2 was found to be correlated with lower overall survival in ovarian cancer patients, which coincides with elevated E-cadherin mRNA levels following siRNA-mediated knockdown of ZEB2 in ovarian cancer cell lines. However, the correlation between ZEB2 and N-cadherin in ovarian cancer remains unclear.

1.2 Transforming growth factor-β superfamily

The transforming growth factor-β (TGF-β) superfamily of ligands regulate pathways important in normal growth and development, as exemplified by the prototypical member TGF-β1, which controls cell proliferation, differentiation, growth, and apoptosis in numerous cell

types (reviewed in [50, 51]). Additionally, the TGF-β superfamily is segmented into several member groups (Table 1) and several individually grouped ligands (Table 2), both with varied roles in normal development.

Table 1. TGF-β superfamily ligand subfamilies and their originally discovered functions.

TGF-β Ligand Subfamily	Example Member	Originally Discovered Function	Reference
TGF-β	TGF-β1	Promotes cell proliferation in non-neoplastic normal rat kidney fibroblast cells	[52]
Activin/Inhibin	Inhibin	Inhibits follicle-stimulating hormone production in murine pituitary cells	[53]
Growth differentiation factor (GDF)	GDF1	Left-right axis and mesoderm formation in mouse embryonic development	[54]
Glial-derived neurotrophic factor (GDNF)	GDNF	Promotes survival of embryonic neurons	[55]
Bone morphogenetic protein (BMP)	BMP2	Promotes *in vivo* bone and cartilage formation in rats	[56, 57]

Table 2. Other TGF-β superfamily ligands and their originally discovered functions.

TGF-β Ligand	Originally Discovered Function	Reference
Müllerian inhibiting substance (MIS)/anti-Müllerian hormone (AMH)	Inhibits Müllerian duct development	[58]
Lefty	Left-right asymmetry determination	[59]
Nodal	Left-right asymmetry determination	[60]

1.2.1 Bone morphogenetic protein

Bone morphogenetic proteins (BMPs) are members of the TGF-β superfamily originally identified as polypeptides involved in bone and cartilage formation [56, 61], and was later implicated in the normal development of other tissues such as cardiac, gastrointestinal, and ovarian (reviewed in [51]). Originally discovered by Urist as a demineralized bone matrix extract that could induce *in vivo* osteogenesis [62, 63], seven individual BMPs (BMP1-BMP7) were cloned and sequenced soon after, six of which (BMP2-BMP7) belong to the TGF-β superfamily [56, 64, 65]. There are now more than 20 BMP family members grouped into several subfamilies based on their amino acid sequence homology [66, 67].

Table 3. Alternative and official names of selected BMP family members. Adapted from [66].

BMP	GDF	Other name(s)	Official symbol
BMP2	-		BMP2
BMP3A	-		BMP3
BMP3B	GDF10		GDF10
BMP4	-		BMP4
BMP5	-		BMP5
BMP6	-		BMP6
BMP7	-		BMP7
BMP8A	-		BMP8A
BMP8B	-		BMP8B
BMP9	GDF2		BDF2
BMP10	-		BMP10
BMP11	GDF11		GDF11
BMP12	GDF7		GDF7
BMP13	GDF6		GDF6
BMP14	GDF5		GDF5
BMP15	GDF9B		BMP15
BMP16	-	NODAL	NODAL
BMP17	-	LEFTY1; LEFTYA	LEFTY1
BMP18	-	LEFTY2; LEFTYB	LEFTY2

1.2.1.1 Bone morphogenetic proteins in cancer

In addition to their involvement in normal function and development, BMPs have been implicated in various cancer types, in both cancer promoting and inhibitory roles (Table 4). Cancer cell response to BMPs have been shown to vary dependent on the specific BMP member—cell proliferation in lung cancer cell lines is enhanced with BMP2 [68], but is inhibited by BMP3B [69] and BMP4 [70], cancer type—cell migration and invasion is inhibited by BMP6 in breast cancer cell lines [71], but is enhanced in prostate cancer cell lines [72, 73], and cell type and/or research group—Shon et al. [74] found that BMP4 inhibits cell migration and invasion in breast cancer cell lines, while Guo et al. [75] found that BMP4 enhanced cell migration and invasion in their breast cancer cell lines.

BMP levels in primary tumour samples (Table 5) are mostly in agreement with their actions in corresponding cell lines (Table 4). For instance, BMP3B inhibits cell proliferation in lung cancer cell lines [69], which corresponds to CpG island methylation and reduced BMP3B mRNA in primary lung cancer specimens [76]. However, there are several cell line studies that do not correspond to BMP levels found in primary tumour specimens. For instance, BMP4 mRNA levels were found to be elevated in primary lung tumour specimens [68], but BMP4 was found to inhibit cell proliferation in lung cancer cell lines [70]. Similarly, BMP6 was found to inhibit cell proliferation, migration, and invasion in breast cancer cell lines [71, 77], but elevated levels of BMP6 mRNA were found in primary breast tumour specimens [78]. These differences may be attributed to differences in subtypes between the cell lines and primary tumours.

Table 4. Bone morphogenetic proteins and their action in lung, breast, and prostate cancer.

Cancer	BMP(s) involved	Action of BMP	Reference
Lung	BMP2	Promotes cell proliferation, migration, and invasion	[68]
	BMP3B	Inhibits cell proliferation	[69]
	BMP4	Inhibits cell proliferation	[70]
	BMP7	Inhibits cell migration and invasion	[79]
Breast	BMP2	Inhibits cell proliferation; promotes cell migration and invasion	[80, 81]
	BMP4	Inhibits cell proliferation; variable cell migration and invasion response	[74, 75]
	BMP6	Inhibits cell proliferation, migration, and invasion	[71, 77]
	BMP7	Variable cell proliferation response; promotes cell migration and invasion	[82, 83]
	BMP9	Inhibits cell proliferation, migration, and invasion	[84]
	BMP10	Inhibits cell proliferation, migration, and invasion	[85]
	BMP15	Inhibits cell invasion	[86]
Prostate	BMP2	Variable cell proliferation response; promotes cell migration and invasion	[72, 87, 88]
	BMP6	Inhibits cell proliferation; promotes cell migration and invasion	[72, 73, 89]
	BMP7	Inhibits cell proliferation; promotes cell migration and invasion	[90, 91]
	BMP9	Inhibits cell proliferation, migration, and invasion	[92]
	BMP10	Inhibits cell proliferation, migration, and invasion	[93]

Table 5. Bone morphogenetic proteins and their clinical relevance in lung, breast, and prostate cancer.

Cancer	BMP(s) involved	Clinical relevance	Reference
Lung	BMP2	Elevated mRNA and protein levels in primary tumour samples	[68]
	BMP3B	CpG island hypermethylation and reduced mRNA levels in primary tumour samples	[76]
	BMP4	Elevated mRNA levels in primary tumour samples	[68]
	BMP7	Reduced mRNA levels correlated with metastasis	[79]
Breast	BMP2	Recombinant human BMP2 inhibited cell proliferation in some primary tumour specimens; decreased mRNA levels in primary tumour samples	[94, 95]
	BMP4	Elevated mRNA levels in primary tumour samples	[78]
	BMP6	Elevated mRNA levels in primary tumour samples	[78]
	BMP7	Elevated mRNA levels in primary tumour samples	[78]
	BMP9	Decreased mRNA levels in primary tumour samples	[84]
	BMP10	Decreased mRNA levels in primary tumour samples	[85]
	BMP15	Decreased mRNA levels in primary tumour samples	[86]
Prostate	BMP2	Variable immunohistochemical staining in primary tumour samples	[96, 97]
	BMP6	Elevated mRNA levels and increased immunohistochemical staining in primary tumour samples	[98]
	BMP7	Elevated mRNA levels in primary tumour samples	[99]
	BMP9	Decreased immunohistochemical staining in primary tumour samples	[92]
	BMP10	Decreased mRNA levels and reduced immunohistochemical staining in primary tumour samples	[93]

1.2.1.1.1 Bone morphogenetic proteins in ovarian cancer

Although the mRNA of numerous BMPs have been detected in several ovarian cancer cell lines [100], the function of BMPs in ovarian cancer is not well studied, with the majority of the literature focusing on the BMP subfamily of BMP2 and BMP4, and limited studies on BMP6. Exogenous BMP6 treatment on primary ovarian cancer cells was found to induce cell spreading and increase cell migration, with no effect on cell proliferation [101]. Likewise, exogenous treatment of BMP4 on primary ovarian cancer cells induced cell spreading and increased cell migration, with no effect on cell proliferation [102, 103]. Additionally, BMP4 treatment altered primary ovarian cancer cell morphology, inducing a cell spreading phenotype with increased cell adhesion [102, 103], and resulted in an EMT response, whereby Snail and Slug mRNA and protein expression increased, with a subsequent decrease in E-cadherin mRNA and protein levels, with corresponding increases in cell migration and invasion [103]. In contrast to primary ovarian cancer cells, which were found to both express BMP4 mRNA and secrete mature BMP4 protein, BMP4 mRNA was detected in several established ovarian cancer cell lines, but secreted protein was undetectable in conditioned media [102]. These studies would suggest that elevated BMP4 expression correlates with a poor prognosis. Interestingly, a separate group found that elevated BMP4 expression to be a positive prognostic factor among their cohort of patients [104].

Elevated BMP2 mRNA expression has been found in primary ovarian cancer cells, and coincides with strong immunohistochemical staining in tumour tissue samples [105]. Subsequent studies inversely correlated BMP2 expression in tumour tissue with survival, and separately shows that exogenous BMP2 treatment on ovarian cancer cell lines increased cell migration [106]. Similar to the lone BMP4 study with contrasting clinical findings, one group

has found a contrasting correlation in their cohort of patients of high BMP2 expression with increased five-year and average survival rates [107].

1.2.1.2 Bone morphogenetic protein 2

BMP2 regulates normal biological functions such as skeletal repair [108] and cardiac development [109]. The subclass of BMPs containing BMP2/4, which is expressed in granulosa and theca cells of human ovarian tissue, promotes folliculogenesis [110] and regulates ovarian steroidogenesis [111]. BMP2 has been implicated in various cancers, although the role of BMP2 is varied depending on cancer type. For instance, BMP2 has been shown to stimulate the growth of pancreatic cancer cell lines and elevated levels are correlated with poorer patient survival [112], and BMP2 was shown to stimulate cell migration and invasion in prostate cancer cell lines [88], while BMP2 has been suggested to act a tumour suppressor in colon cancer cells [113].

BMP2 was found to be over-expressed in primary cultures of epithelial ovarian cancer cells via microarray profiling [105] and exogenous treatment of BMP2 was found to stimulate cell motility in ovarian cancer cell lines [106]. An examination of eight primary epithelial ovarian cancer samples using ELISA detected mature BMP2 protein in the culture media at an approximate range of 0-1000 pg/mL [106]. It was determined that elevated BMP2 protein expression was significantly correlated with decreased survival time in advanced stage ovarian cancer patients via a tissue microarray [106]. Despite the correlation between BMP2 and poor ovarian cancer patient outcomes, little is known about the molecular mechanism of how BMP2 induces ovarian cancer cell motility.

BMP2 has been implicated in enhancing cell motility and invasiveness via the SMAD-independent PI3K/Akt pathway in gastric cancer cells [114], and enhancing motility in

chondrosarcoma cells [115]. Additionally, BMP2-induced morphological changes mediated by an EMT have been described in gastric [114] and colon cancer cell lines [116]. BMP4, a BMP in the same group as BMP2, has been shown to induce EMT-mediated morphological changes in ovarian cancer cells and was found to exhibit increased cell motility and invasiveness [103]. However, the role of BMP2 in ovarian cancer cell motility and invasiveness, and the ability of BMP2 to induce morphological changes mediated by EMT is not well characterized.

1.2.2 Signal transduction

TGF-β signal transduction is mediated through binding of a TGF-β superfamily of ligands to a type II serine/threonine kinase receptor (ACVR2A, ACVR2B, AMHR2, BMPR2, or TGFBR2) that complexes with a type I serine/threonine kinase receptor (ALK1-7) (Figure 1; reviewed in [51, 117]). This hetero-dimerization of the type I and type II receptor induces the transphosphorylation of the type I receptor by the type II receptor, which results in an activated complex that phosphorylates receptor-regulated SMADS (R-SMADs) (Figure 1; reviewed in [51, 117]). Activated R-SMADs associate with a common SMAD (co-SMAD; SMAD4) and the R-SMAD:co-SMAD complex translocates to the nucleus to regulate DNA transcription (Figure 1; reviewed in [51, 117]).

The activated TGF-β type I receptors ALK1, ALK2, ALK3, and ALK6 are known to activate SMAD1, SMAD5, and SMAD8 via phosphorylation, while ALK4, ALK5, and ALK7 are known to activate SMAD2 and SMAD3 via phosphorylation (Figure 1; reviewed in [51, 117]). It has been previously described that BMP2 induces SMAD1/5/8 phosphorylation and does not induce SMAD2/3 phosphorylation (reviewed in [51, 117]).

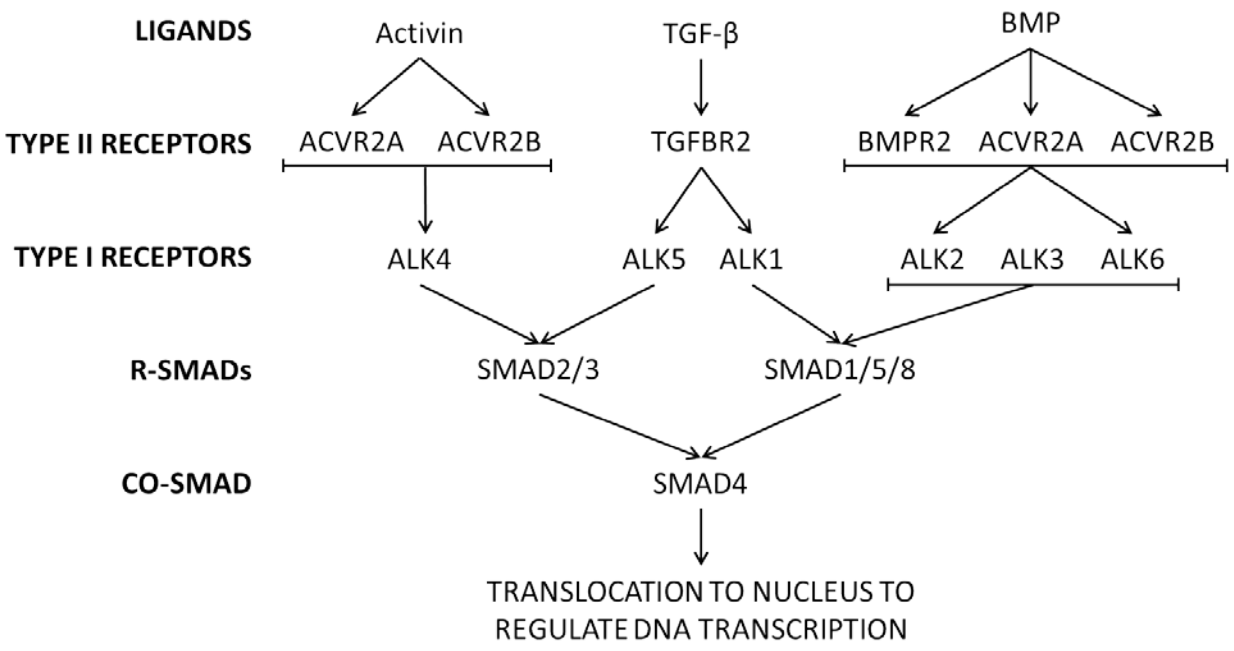

Figure 1. TGF-β signal transduction. Adapted from [51].

1.3 Hypotheses and objectives

The hypothesis of this study is:

BMP2 stimulates ovarian cancer cell motility and is mediated through differential expression of cadherin adhesion molecules induced via activation of the SMAD-dependent pathway.

The specific objectives of this work are:

Objective 1: To determine the relative expression of TGF-β type I receptors in ovarian cancer cell lines, and to determine if BMP2 can stimulate ovarian cancer cell motility.

Objective 2: To determine if cadherins are involved in BMP2-induced ovarian cancer cell migration.

Objective 3: To determine if BMP2 can activate the SMAD-dependent pathway, and whether activation is required for the observed effects on cadherin proteins and mRNA expression in ovarian cancer cell lines.

Chapter 2: Materials and Methods

2.1 Cell culture

SKOV3 human ovarian cancer cell lines were obtained from American Type Culture Collection (Manassas, VA, USA). OVCAR8 and OVCAR5 human ovarian cancer cell lines were kindly provided by Dr. T.C. Hamilton (Fox Chase Cancer Center, Philadelphia, PA). Cells were cultured in a 1:1 (v/v) mixture of Medium 199/MCDB105 medium (Sigma–Aldrich, Oakville, ON) containing 5% fetal bovine serum (FBS; Hyclone Laboratories Inc., Logan, UT), 100 U/mL penicillin G and 100 g/mL streptomycin (Life Technologies, Inc., Rockville, MD). Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 to 95% air.

2.2 Antibodies and reagents

Polyclonal rabbit anti-actin (C-11; sc-1615) and polyclonal rabbit SMAD1/5/8 (N-18; sc-6031-R) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), as was the horseradish peroxidase-conjugated donkey anti-goat IgG (sc-2020). Polyclonal rabbit anti-phospho-SMAD1 (Ser463/465)/SMAD5 (Ser463/465)/ SMAD8 (Ser426/428) (#9511), monoclonal mouse anti-SMAD2 (L16D3; #3103), polyclonal rabbit anti-phospho-SMAD2 (Ser465/467; #3101), monoclonal rabbit anti-SMAD3 (C67H9; #9523), and monoclonal anti-phospho-SMAD3 (Ser423/425; #9520) antibodies were purchased from Cell Signaling Technology (Beverly, MA). Monoclonal anti-E-cadherin (#610181) and anti-N-cadherin (#610920) antibodies were purchased from BD Biosciences (Mississauga, ON). Horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit IgGs were purchased from Bio-Rad Laboratories (Hercules, CA). Recombinant human BMP2 (355-BM), 6-[4-[2-(1-Piperidinyl)ethoxy]phenyl]-3-(4-pyridinyl)-pyrazolo[1,5-a]pyrimidine dihydrochloride

(dorsomorphin dihydrochloride; #3093) and 4-[6-[4-(1-Methylethoxy)phenyl]pyrazolo[1,5-a]pyrimidin-3-yl]-quinoline (DMH-1; #4126) were purchased from R&D Systems (Minneapolis, MN). 4-(5-Benzol[1,3]dioxol-5-yl-4-pyrldin-2-yl-1H-imidazol-2-yl)-benzamide hydrate (SB-431542; S4317) was purchased from Sigma-Aldrich Corp. (Oakville, ON).

2.3 Reverse transcription quantitative real-time PCR (RT-qPCR)

Cells were washed with cold PBS and total RNA was extracted using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription was performed with 1 μg RNA, random primers, and M-MLV reverse transcriptase (Promega, Madison, WI). Each 20 μL RT-qPCR reaction contained 1× SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) 25 ng cDNA and 250 nM of each specific primer. The following primers were used for SYBR Green RT-qPCR amplification: E-cadherin, 5′-ACA GCC CCG CCT TAT GAT T-3′ (sense) and 5′-TCG GAA CCG CTT CCT TCA-3′ (antisense); N-cadherin, 5'-GGA CAG TTC CTG AGG GAT CA-3'; SNAIL, 5′-CCC CAA TCG GAA GCC TAA CT-3′ (sense) and 5′-GCT GGA AGG TAA ACT CTG GAT TAG A-3′ (antisense); SLUG, 5′-TTC GGA CCC ACA CAT TAC CT-3′ (sense) and 5′-GCA GTG AGG GCA AGA AAA AG-3′ (antisense); TWIST1, 5'-GGA GTC CGC AGT CTT ACG AG-3' (sense) and 5'-TCT GGA GGA CCT GGT AGA GG-3' (antisense); ZEB1, 5'-GCA CCT GAA GAG GAC CAG AG-3' (sense) and 5'-TGC ATC TGG TGT TCC ATT TT-3' (antisense); and GAPDH, 5′-GAG TCA ACG GAT TTG GTC GT-3′ (sense) and 5′-GAC AAG CTT CCC GTT CTC AG-3′ (antisense). The specificity of each assay was validated by dissociation curve analysis and agarose gel electrophoresis of PCR products. Assay performance was validated by evaluating amplification efficiencies by means of calibration curves, and ensuring that the plot of log input amount vs.

ΔCq has a slope < |0.1|. Alternatively, TaqMan gene expression assays for activin receptor-like kinase (ALK)2, ALK3, ALK4, ALK5, and ALK6 (Hs00153836_m1, Hs01034913_g1, Hs00244715_m1, Hs00610320_m1, and Hs00176144_m1, respectively; Applied Biosystems) were performed on corresponding cDNA samples. Each 20 μL TaqMan reaction contained,112.5 ng cDNA, 1× TaqMan Gene Expression Master Mix (Applied Biosystems), and 1× TaqMan gene expression assay (containing primers and probe). The PCR parameters were 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The comparative Cq ($2^{-\Delta\Delta Cq}$) method with GAPDH as the reference gene was used to determine relative mRNA levels.

2.4 Western blot analysis

Cells were lysed in lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μM aprotinin, 1 μM leupeptin and 1 mM PMSF; Cell Signaling Technology, Danvers, MA) and protein concentrations were determined using the DC Protein Assay kit with BSA as the standard (Bio-Rad Laboratories). Equal amounts of protein were separated by SDS polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes. After blocking with Tris-buffered saline containing 5% non-fat dry milk or BSA (depending on antibody manufacturer instructions), membranes were incubated overnight at 4 °C with primary antibodies followed by incubation with HRP-conjugated secondary antibodies. Immunoreactive bands were detected with Enhanced Chemiluminescent Substrate (ECL) or SuperSignal West Femto Chemiluminescent Substrate (Pierce, Rockford, IL). Membranes detecting phosphorylated targets were stripped with stripping buffer at 50 °C for 30 min and re-probed with the appropriate

non-phosphorylated primary antibodies. Membranes were probed with anti-actin as a loading control. Quantitative densitometry was performed using Adobe Photoshop (Adobe Systems, San Jose, CA) and normalized to respective levels of total SMADs for experiments examining phosphorylated SMADs, or actin for experiments examining non-SMAD targets.

2.5 Transwell migration assay

Migration assays were performed in modified Boyden chambers as described in [118] with minor modifications. Cell culture inserts (24-well, 8-μm pore size; BD Biosciences) were seeded with 3.75×10^4 SKOV3, 7.5×10^4 OVCAR5, or 4.5×10^4 OVCAR8 in 250 μL culture medium containing 0.1% FBS. 750 μL of culture medium containing 1% (SKOV3) or 5% (OVCAR5 and OVCAR8) FBS was added to the lower chamber and served as a chemotactic agent. Cells that penetrated the membrane were fixed with cold methanol (-20°C) and air dried. Cell nuclei were stained with Hoechst 33258 (Sigma-Aldrich) and counted by epifluorescence microscopy with Northern Eclipse 6.0 software (Empix Imaging, Mississauga, ON). Triplicate inserts were used for each individual experiment, and five microscopic fields were counted per insert.

2.6 Small interfering RNA (siRNA) transfection

To knockdown endogenous ALK2, ALK3, ALK6, SMAD2, SMAD3 and N-cadherin, cells were transfected with 25 nM (50 nM for SMAD3) ON-TARGETplus SMARTpool siRNA targeting that specific gene (Dharmacon Research, Inc., Lafayette, CO) or siCONTROL NON-TARGETING pool siRNA (transfection control, siCtrl; Dharmacon) using Lipofectamine

RNAiMAX (Invitrogen, Burlington, ON). Knockdown efficiency was examined by RT–qPCR or Western blot analysis.

2.7 Statistical analysis

Results were analyzed by one-way ANOVA followed by Student–Newman–Keuls's multiple comparison *post hoc* test. Results are presented as the mean ± SEM of at least three independent experiments and data were considered significantly different from each other if $p < 0.05$.

Chapter 3: Results

3.1 Differential mRNA levels of TGF-β type I receptors and BMP2 in OVCAR8, OVCAR5, and SKOV3 cells

As a first step towards analyzing the role of BMP2 in ovarian cancer cell migration, I examined the expression profiles of TGF-β (ALK4/5) and BMP (ALK2/3/6) type I receptors in OVCAR8, OVCAR5 and SKOV3 cell lines prior to confirming the activation of canonical BMP signaling. My results show a similar mRNA expression pattern of ALK2/3/4/5 across all three, cell lines, whereby ALK2/3/5 is expressed at a significantly greater level than ALK4 (Figure 2). SKOV3 was found to have a higher relative mRNA expression of ALK6 compared to OVCAR8 and OVCAR5 (Figure 2).

Additionally, I examined the baseline mRNA expression of BMP2 in OVCAR8, OVCA5, and SKOV3. My results show that SKOV3 and OVCAR5 have a high basal expression of BMP2 mRNA relative to OVCAR8 (Figure 3).

3.2 BMP2 induces EMT-like changes in some ovarian cancer cell lines

Next, I investigated the functional role of BMP2 in ovarian cancer cell lines. OVCAR5 and SKOV3 cells exhibited enhanced transwell cell migration following treatment with BMP2 for 72 h, whereas OVCAR8 cells did not (Figure 4). Similarly, OVCAR8 cells did not exhibit any morphological changes following 72-h treatment with BMP2 (Figure 5A), whereas exogenous BMP2 stimulation of SKOV3 cells induced transformation of the organized cuboidal cell colonies to a leaner phenotype with multiple spindle-like projections and irregular colony packing (Figure 5B).

As the morphological and functional changes exhibited by SKOV3 cells upon BMP2 stimulation were consistent with EMT, I next examined the molecular profiles of EMT-associated cadherin proteins and their transcription factors. BMP2 was found to down-regulate E-cadherin and up-regulate N-cadherin protein levels in a time-dependent manner in SKOV3 cells after daily exogenous BMP2 treatment for 72 h, with significant effects observed at 72 h for both N-cadherin and E-cadherin (Figure 6). In contrast, treatment with BMP2 for 72 h significantly increased E-cadherin and decreased N-cadherin protein levels in OVCAR8 cells (Figure 7). E-cadherin protein levels were not significantly altered following treatment with BMP2 in OVCAR5 cells (N-cadherin protein levels were undetectable in this cell line; Figure 8).

To investigate whether the changes in E-cadherin and N-cadherin in SKOV3 cells are mediated by transcriptional repressors of E-cadherin or inducers of N-cadherin, RT-qPCR was used to examine the mRNA levels of ZEB1, SNAIL, SLUG, and TWIST1 following treatment with BMP2 for varying amounts of time (1, 3, 6 or 24 h). My data suggests that altered expression of SNAIL, SLUG and possibly ZEB1, but not TWIST1, may contribute to these EMT-like changes in SKOV3 cells (Figure 9). In particular, SLUG mRNA levels were significantly up-regulated at 6 and 24 h, whereas mRNA levels of SNAIL were increased at 1 and 24 h (Figure 9). The mRNA levels of E-cadherin and N-cadherin were also examined at the same time points and found E-cadherin mRNA to be significantly down-regulated at 24 h, but no significant change in N-cadherin mRNA levels was detected (Figure 9).

3.3 BMP2 induces SMAD1/5/8 phosphorylation, and differentially induces SMAD2 and SMAD3 phosphorylation in SKOV3, OVCAR8, and OVCAR5 cells

Consistent with published reports of canonical SMAD signaling through SMAD1/5/8 upon BMP2 stimulation [51, 117], BMP2 was found to induce SMAD1/5/8 phosphorylation in SKOV3, OVCAR8 and OVCAR5 cells (Figure 10, Figure 11, and Figure 12, respectively). Additionally, BMP2 induced SMAD2 and SMAD3 phosphorylation in SKOV3 cells, with maximal effects at 60 and 30 min, respectively (Figure 10). In contrast, treatment of OVCAR8 cells with BMP2 reduced the phosphorylation of SMAD3, while not significantly altering SMAD2 phosphorylation levels (Figure 11). In OVCAR5 cells, phospho-SMAD2 levels were unchanged whereas there tended to be an increase in the levels of phospho-SMAD3 following treatment with BMP2 (Figure 12).

3.4 ALK3 mediates BMP2-induced SMAD2 and SMAD3 phosphorylation in SKOV3 cells

Pharmacological inhibitors of TGF-β and BMP type I receptors were then used to investigate the relative contributions of the two receptor classes to the BMP2-mediated increases in SMAD2 and SMAD3 phosphorylation in SKOV3 cells. Prior to stimulation with BMP2, SKOV3 cells were pretreated for 1 h with the TGF-β type I receptor inhibitor SB-431542 (inhibitor of ALK4, ALK5, and ALK7; [119]) or the BMP type I receptor inhibitors Dorsomorphin (inhibitor of ALK2, ALK3, and ALK6; [120]) and DMH-1 (inhibitor of ALK2 and ALK3; [121]). Dorsomorphin or DMH-1, but not SB-431542, blocked BMP2-induced SMAD2 phosphorylation (Figure 13). Similarly, pretreatment for 1 h with Dorsomorphin or DMH-1, but not SB-431542, blocked BMP2-induced SMAD3 phosphorylation (Figure 14).

The effectiveness of Dorsomorphin and DMH-1 pretreatment in blocking BMP2-induced SMAD2/3 phosphorylation suggests involvement of ALK2 and ALK3. To determine which of these ALKs contribute to BMP2-induced SMAD2/3 phosphorylation and to confirm my results using pharmacological inhibitors, I used a siRNA-mediated approach to knockdown ALK2, ALK3, and ALK6 prior to BMP2 treatment. I found that siRNA-mediated knockdown of ALK3, but not ALK2 or ALK6 ablated the BMP2-induced phosphorylation of SMAD2 and SMAD3 (Figure 15).

3.5 TGF-β type I and BMP type I receptors mediate the BMP2-induced E-cadherin up-regulation and N-cadherin down-regulation in SKOV3 cells

I next employed the pharmacological inhibitors of TGF-β and BMP type I receptors to determine whether certain groups of ALKs are involved in the observed alterations in E-cadherin and N-cadherin in SKOV3 cells. SKOV3 cells were pretreated with SB-431542, Dorsomorphin, or DMH-1 for 1 h prior to treatment with BMP2 for 72 h. Interestingly, BMP2-induced down-regulation of E-cadherin was blocked by SB-431542 (Figure 16) and unaffected by Dorsomorphin (Figure 17) or DMH-1 (Figure 18). Conversely, the BMP2-induced up-regulation of N-cadherin was blocked by SB-431542 (Figure 19), Dorsomorphin (Figure 20), and DMH-1 (Figure 21).

3.6 DMH-1 and Dorsomorphin, but not SB-431542, abolishes BMP2-induced transwell migration in SKOV3 cells

Although SB-431542 was able to block the BMP2-induced E-cadherin to N-cadherin switch in SKOV3 cells, it was unable to block BMP2-induced transwell migration (Figure 22).

In contrast, co-treatment with either DMH-1 or Dorsomorphin abolished BMP2-induced transwell migration (Figure 22). Interestingly, unlike DMH-1, treatment with Dorsomorphin alone significantly suppressed basal SKOV3 cell migration (Figure 22).

3.7 SMAD2 and SMAD3 mediate BMP2-induced transwell migration in SKOV3 cells

Given that BMP2 induces SMAD1/5/8 is phosphorylation in SKOV3, OVCAR5, and OVCAR8, only SKOV3 exhibited an increase in transwell migration and phosphorylation of both SMAD2/3, these data suggests that SMAD2 or SMAD3 may be mediating the observed BMP2-induced responses. Therefore, siRNA was used to knockdown SMAD2 and SMAD3 to examine their involvement in BMP2-induced transwell migration. Interestingly, siRNA-mediated knockdown of SMAD2 and SMAD3 revealed that they are independently necessary for BMP2-induced transwell migration in SKOV3 cells (Figure 23). Moreover, knockdown of SMAD3, either alone or in conjunction with SMAD2 knockdown, significantly reduced basal SKOV3 cell migration (Figure 23).

3.8 N-cadherin is required for BMP2-induced transwell migration in SKOV3 cells

To determine whether the up-regulation of N-cadherin is involved in BMP2-induced SKOV3 cell migration, I evaluated the migratory capacity of BMP2-treated SKOV3 cells following siRNA-mediated knockdown of N-cadherin. As shown in Figure 24, down-regulation of N-cadherin abolished BMP2-induced transwell cell migration, but did not alter basal migration.

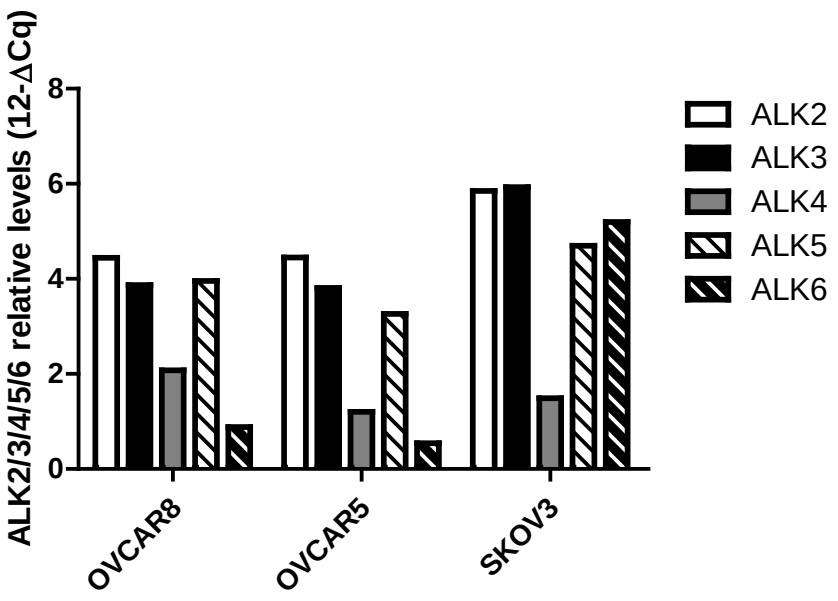

Figure 2. Expression of TGF-β type I serine/threonine kinase receptors in OVCAR8, OVCAR5, and SKOV3 cell lines.
Baseline mRNA levels of TGF-β type I receptors were examined in ovarian cancer cell lines by TaqMan RT-qPCR with GAPDH as the reference gene. Results show the ΔCq values subtracted from 12 from one experiment.

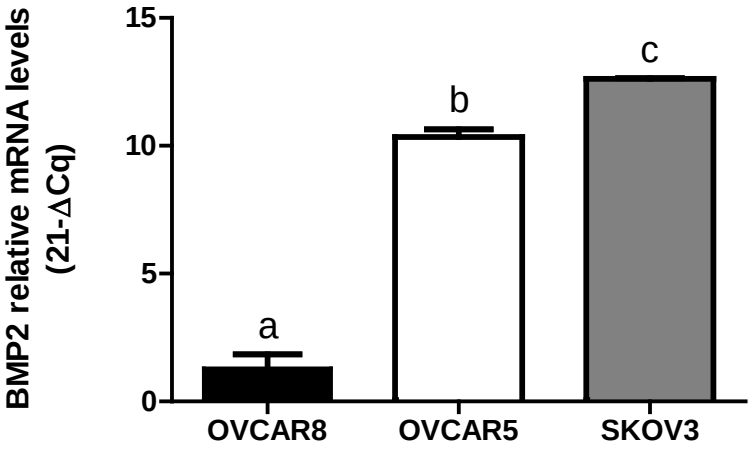

Figure 3. Expression of BMP2 mRNA in OVCAR8, OVCAR5, and SKOV3 cell lines. Baseline mRNA levels of BMP2 was examined in ovarian cancer cell lines by TaqMan RT-qPCR with GAPDH as the reference gene. Results show the ΔCq values subtracted from 21 and represent the mean ± SEM of three independent experiments. Data were analyzed by one-way ANOVA followed by the Newman-Keuls multiple comparison test. Values without a common letter are significantly different ($P < 0.05$).

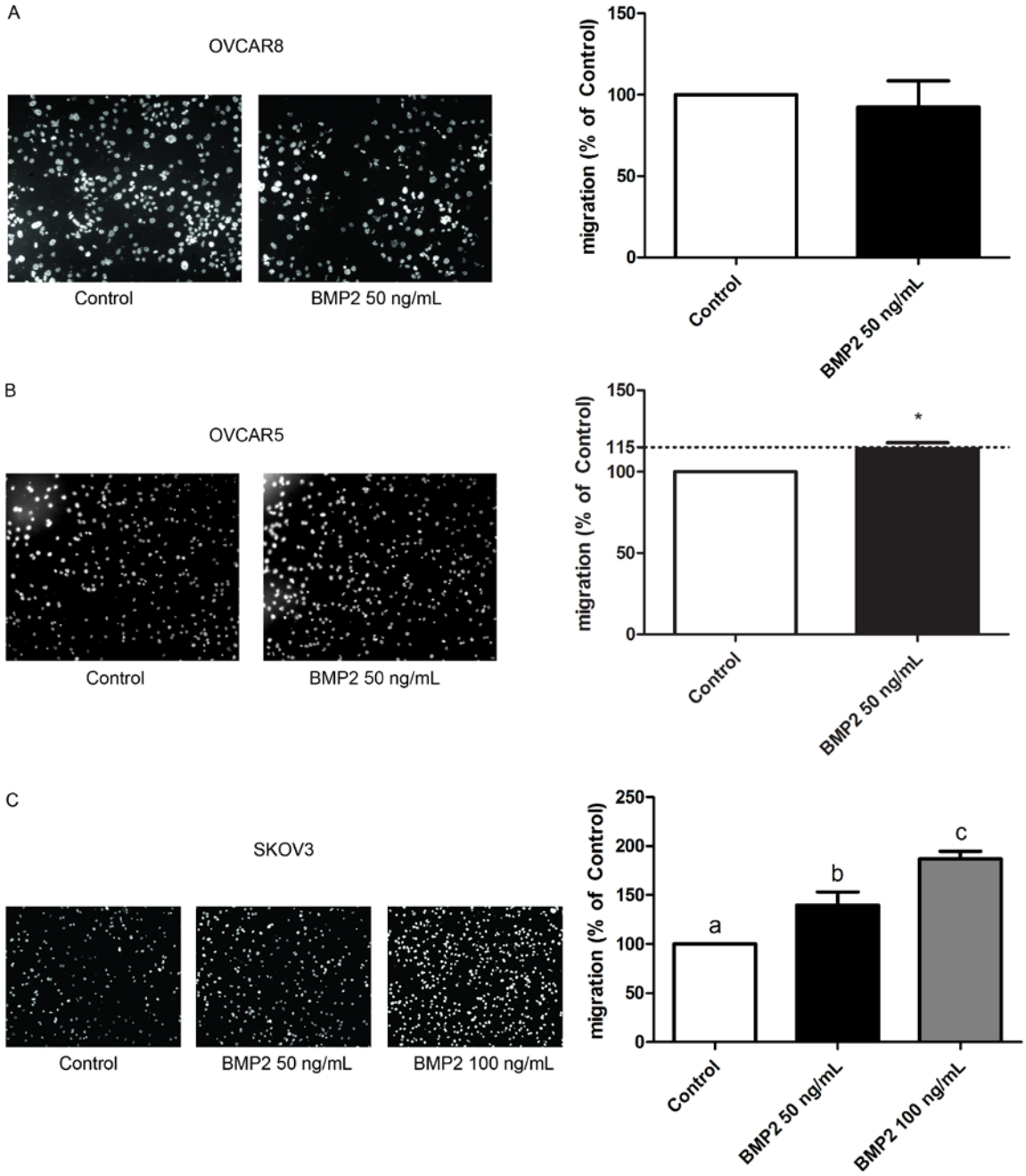

Figure 4. Effect of BMP2 on OVCAR8, OVCAR5, and SKOV3 cell line migration. (A) OVCAR8 and (B) OVCAR5 cells were treated with vehicle control or 50 ng/mL BMP2 every 24 h for 72 h and seeded in Transwell inserts for 48 or 7 h, respectively. (C) SKOV3 cells were treated with vehicle control, 50 ng/mL or 100 ng/mL BMP2 every 24 h for 72 h and seeded in Transwell inserts for 5 h. Results represent the mean ± SEM of three independent experiments. OVCAR5 and OVCAR8 data were analyzed by Student's *t*-test (*$P < 0.05$). SKOV3 data were analyzed by one-way ANOVA followed by the Newman-Keuls multiple comparison test. Values without a common letter are significantly different ($P < 0.05$).

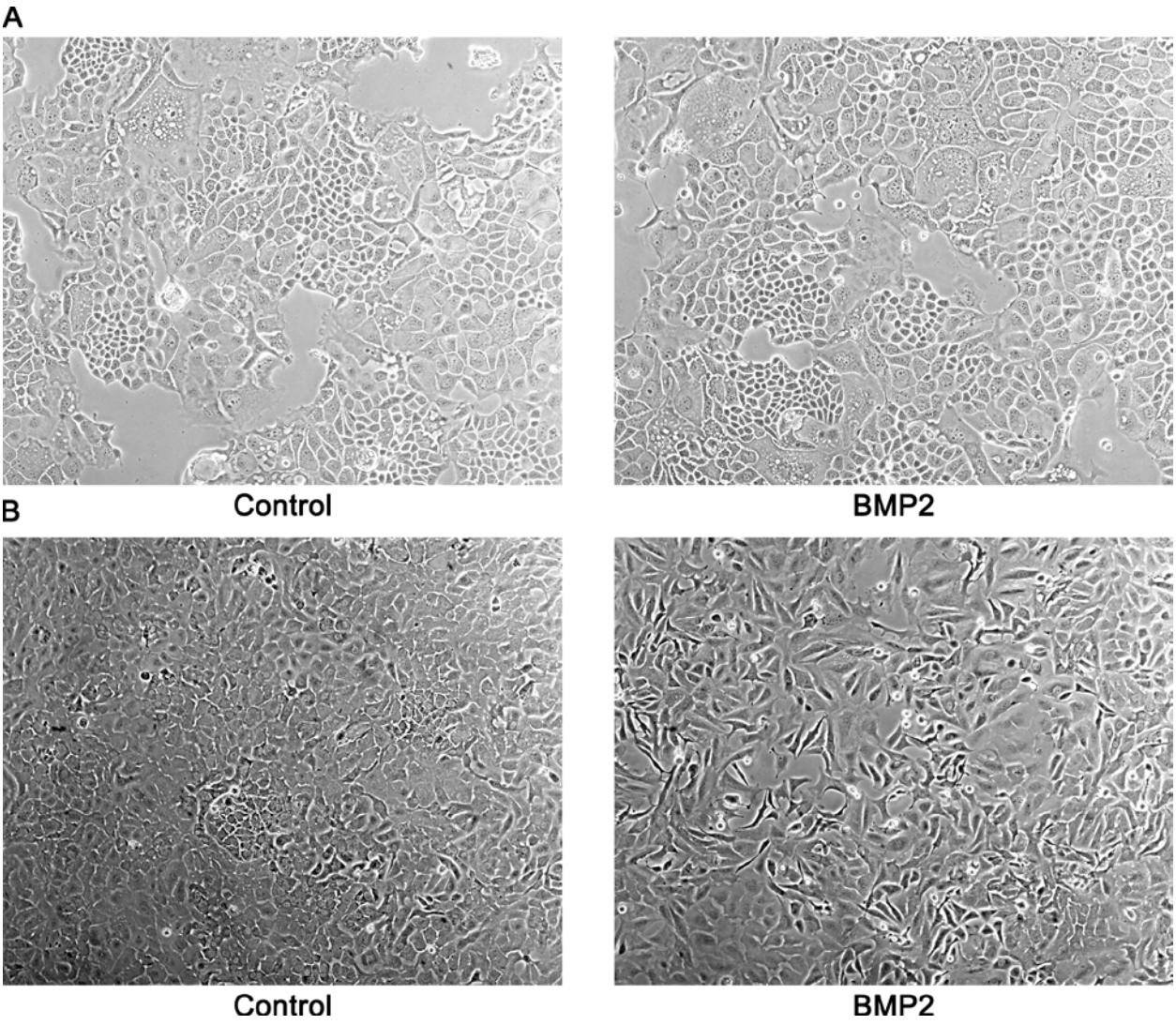

Figure 5. The comparative effects of BMP2 on morphological changes in OVCAR8 and SKOV3 cells.

OVCAR8 (A) or SKOV3 (B) cells were treated with vehicle control or 50 ng/mL BMP2 every 24 h for 72 h and visualized at 72 h. Cells were observed by phase-contrast microscopy and representative micrographs are shown.

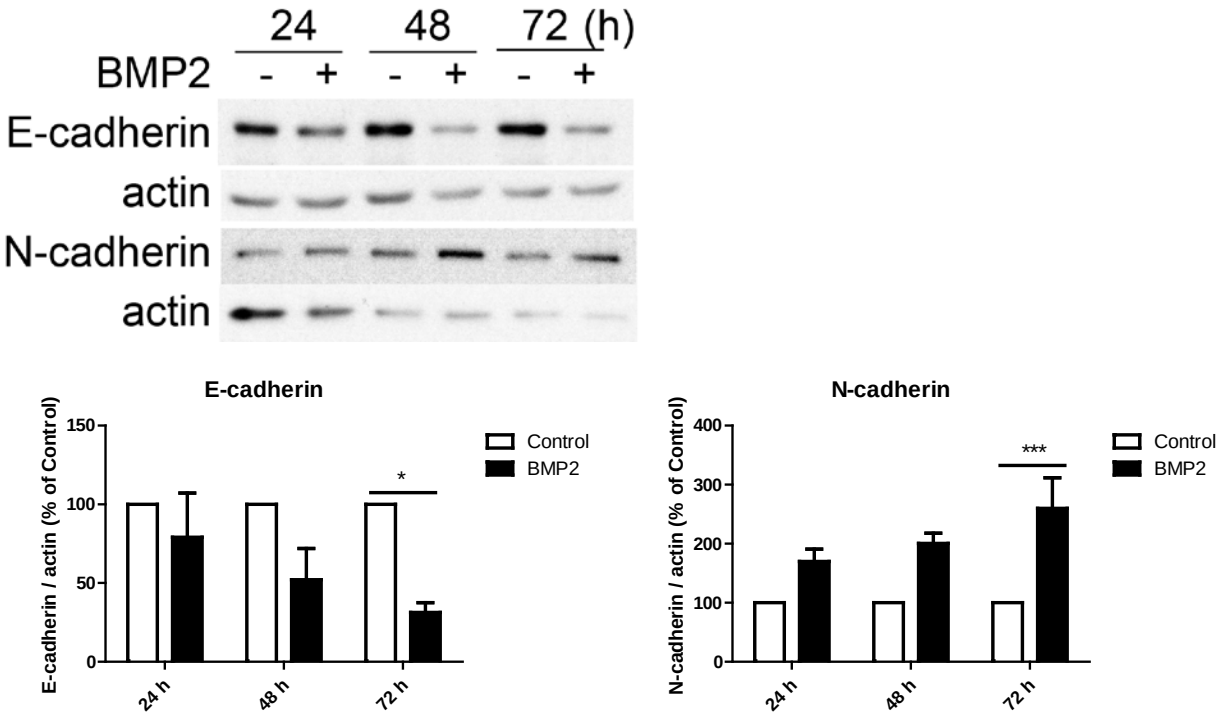

Figure 6. BMP2 down-regulates E-cadherin and up-regulates N-cadherin protein levels in a time-dependent manner in SKOV3.
SKOV3 cells were treated with vehicle control or 50 ng/mL BMP2 for 24, 48, and 72 h. E-cadherin and N-cadherin protein levels were analyzed by Western blotting. The actin antibody was used as a loading control. The immunoblot shown is representative of at least three independent experiments. Cumulative results for quantitative densitometry of the experiments are shown in the lower panel. Results represent the mean ± SEM of the experiments. Data were analyzed by one-way ANOVA followed by the Newman-Keuls multiple comparison test (*$P < 0.05$; ***$P < 0.0001$).

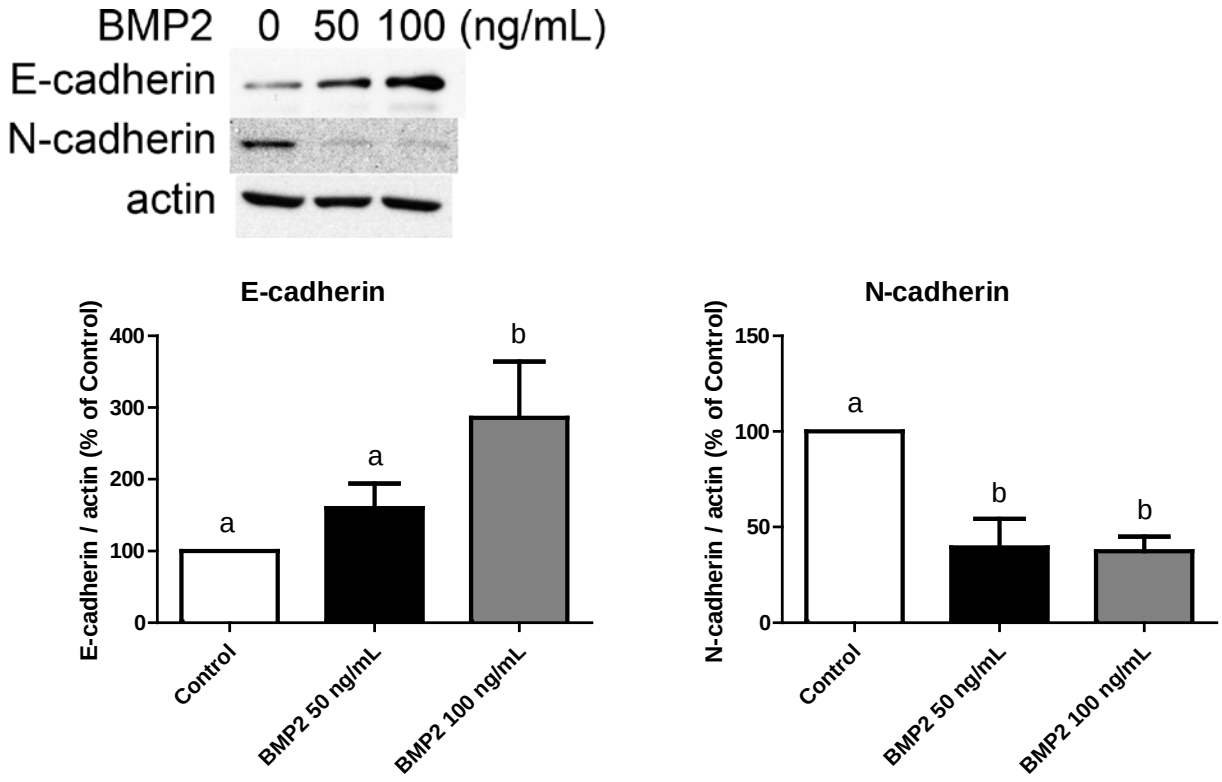

Figure 7. BMP2 up-regulates E-cadherin and down-regulates N-cadherin protein levels in a dose-dependent manner in OVCAR8.
OVCAR8 cells were treated with vehicle control or increasing concentrations of BMP2 for 72 h. E-cadherin and N-cadherin protein levels were analyzed by Western blotting. The actin antibody was used as a loading control. The immunoblot shown is representative of at least three independent experiments. Cumulative results for quantitative densitometry of the experiments are shown in the lower panel. Results represent the mean ± SEM of the experiments. Data were analyzed by one-way ANOVA followed by the Newman-Keuls multiple comparison test. Values without a common letter are significantly different ($P < 0.05$).

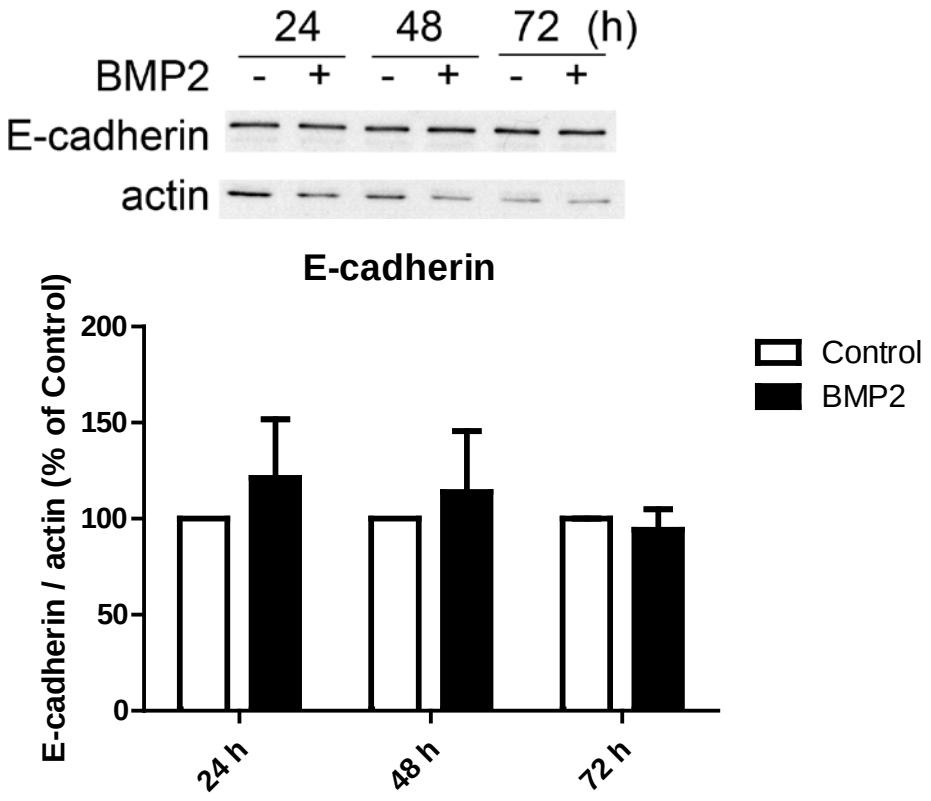

Figure 8. BMP2 does not affect E-cadherin protein levels in OVCAR5 cells.
OVCAR5 cells were treated with vehicle control or 50 ng/mL BMP2 for 24, 48, and 72 h. E-cadherin protein levels were analyzed by Western blotting. The actin antibody was used as a loading control. The immunoblot shown is representative of four independent experiments. Cumulative results for quantitative densitometry of the experiments are shown in the lower panel. Results represent the mean ± SEM of the experiments. Data were analyzed by one-way ANOVA followed by the Newman-Keuls multiple comparison test ($P < 0.05$).

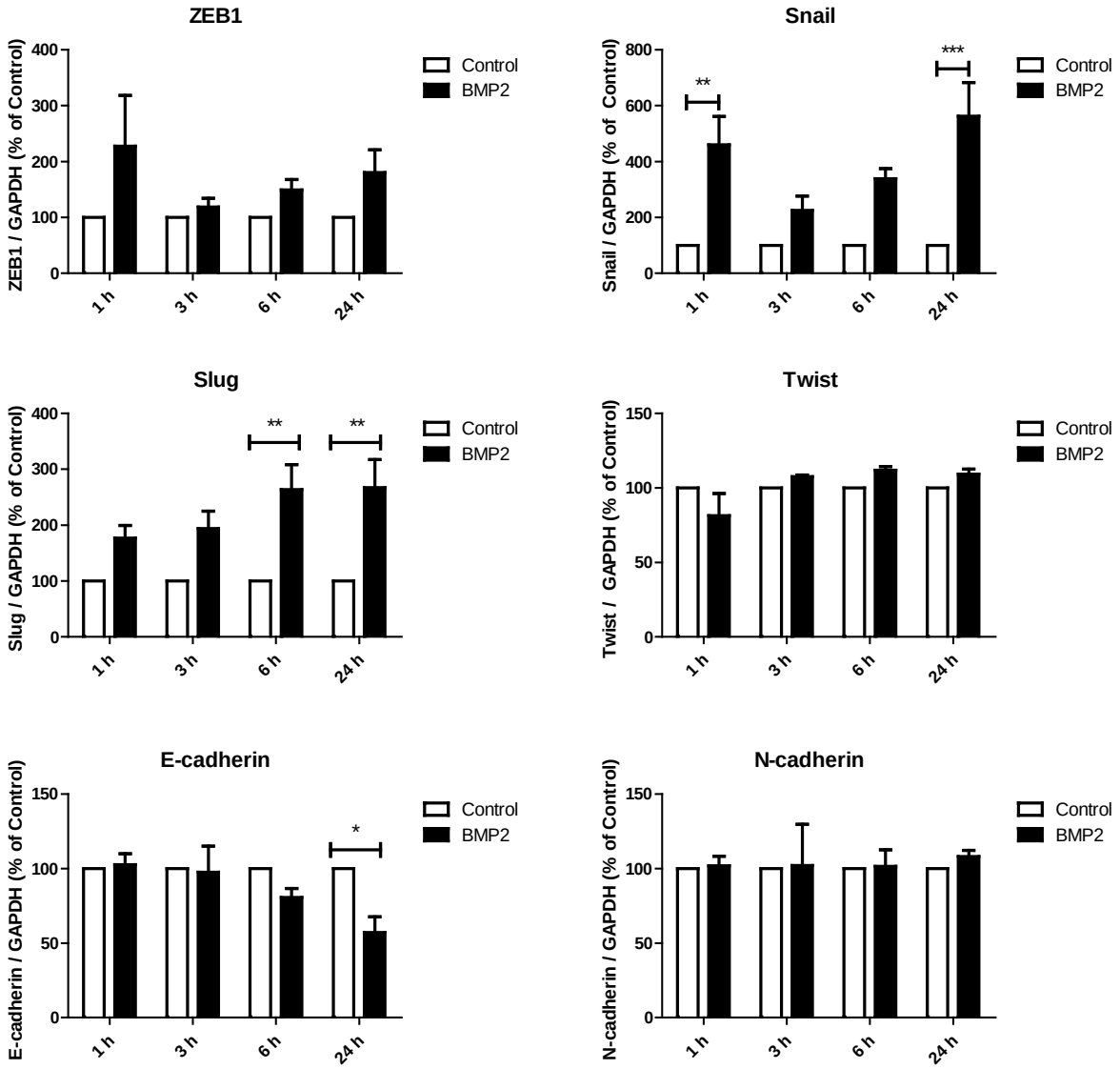

Figure 9. Time-dependent effects of BMP2 on mRNA levels of cell-cell adhesion molecules and their transcription factors in SKOV3.

SKOV3 cells were treated with vehicle control or 50 ng/mL BMP2 for 1, 3, 6, and 24 h. The relative mRNA levels of ZEB1, Snail, Slug, Twist, E-cadherin, and N-cadherin were analyzed by RT-qPCR with GAPDH as the reference gene using the comparative Cq ($2^{-\Delta\Delta Cq}$) method. Cumulative results of at least three independent experiments are shown. Results represent the mean ± SEM of the experiments. Data were analyzed by one-way ANOVA followed by the Newman-Keuls multiple comparison test (*P < 0.05; **P < 0.01; ***P < 0.0001).

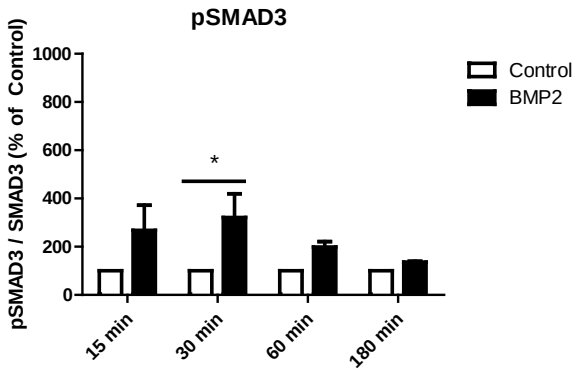

Figure 10. BMP2 induces SMAD1/5/8, SMAD2, and SMAD3 phosphorylation in SKOV3. SKOV3 cells were treated with vehicle control or 50 ng/mL BMP2 for 15, 30, 60, and 180 min as indicated. Phosphorylated and total SMAD1/5/8, SMAD2, and SMAD3 were analyzed by Western blotting. The immunoblot shown in the upper left panel is representative of four independent experiments. Cumulative results for quantitative densitometry of the experiments are shown in the remaining panels. Results represent the mean ± SEM of the experiments. pSMAD1/5/8 data were analyzed by Student's *t*-test (***P < 0.0001). pSMAD2 and pSMAD3 data were analyzed by one-way ANOVA followed by the Newman-Keuls multiple comparison test (*P < 0.05; **P < 0.01).

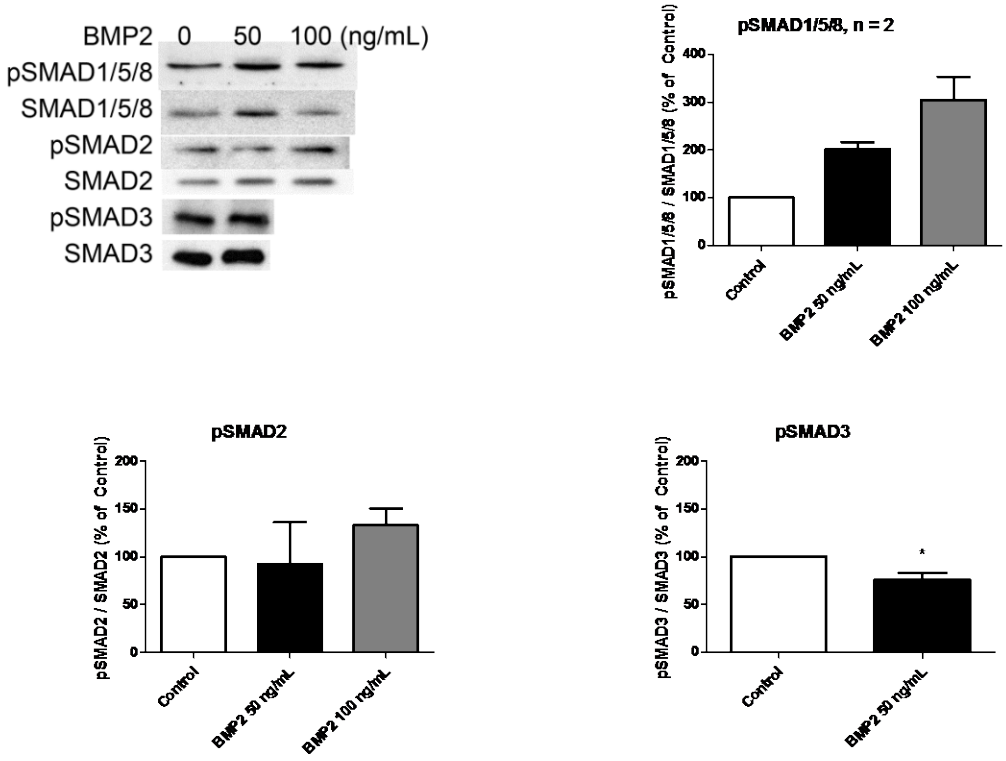

Figure 11. BMP2 induces SMAD1/5/8 phosphorylation, while repressing SMAD3 phosphorylation, and does not affect SMAD2 phosphorylation in OVCAR8.
OVCAR8 cells were treated with vehicle control or 50 ng/mL or 100 ng/mL BMP2 as indicated for 30 min. Phosphorylated and total SMAD1/5/8, SMAD2, and SMAD3 were analyzed by Western blotting. The immunoblot shown in the upper left panel is representative of three independent experiments (except for pSMAD1/5/8; n = 2). Cumulative results for quantitative densitometry of the experiments are shown in the remaining panels. Results represent the mean ± SEM of the experiments. pSMAD2 data were analyzed by one-way ANOVA followed by the Newman-Keuls multiple comparison test ($P > 0.05$). pSMAD3 data were analyzed by Student's *t*-test ($^*P < 0.05$).

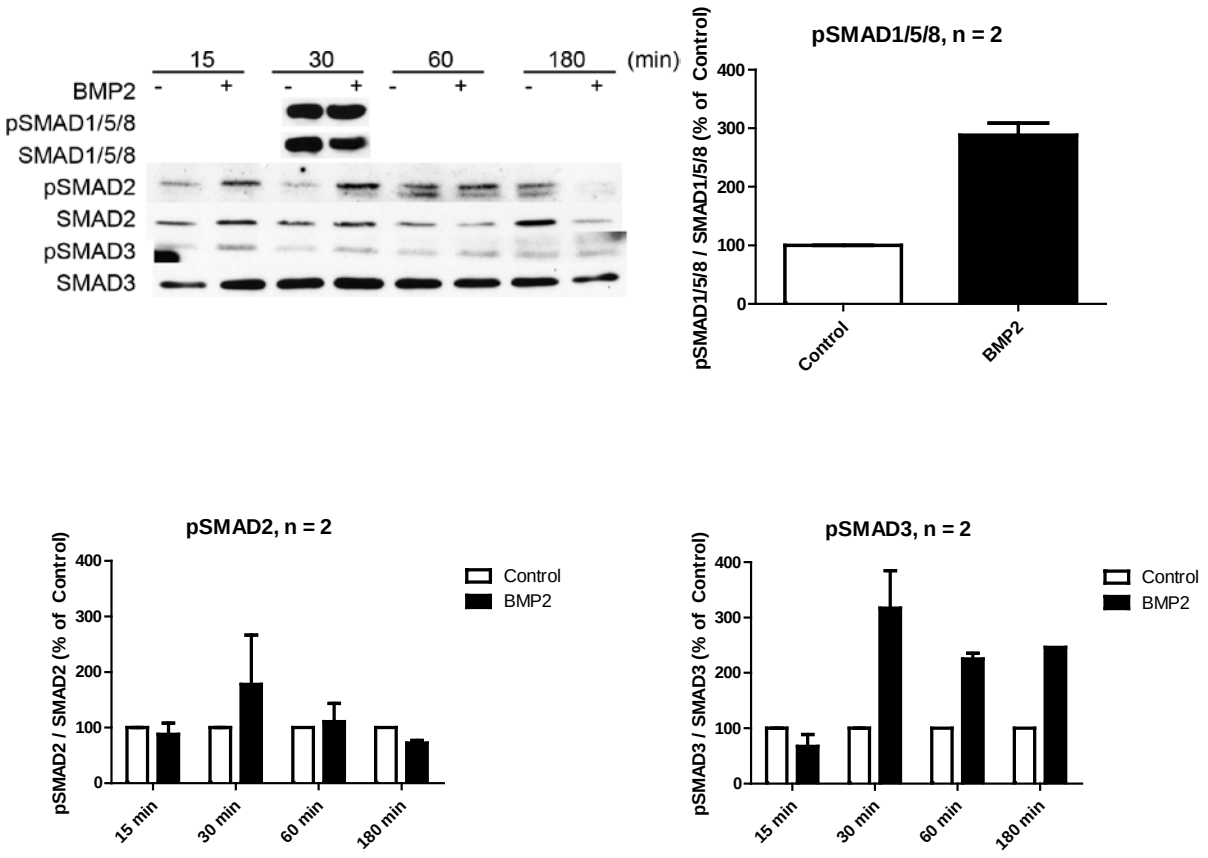

Figure 12. BMP2 may induce SMAD1/5/8 and SMAD3 phosphorylation, but not SMAD2 phosphorylation, in OVCAR5.

OVCAR5 cells were treated with vehicle control or 50 ng/mL BMP2 for 15, 30, 60, or 180 min as indicated. Phosphorylated and total SMAD1/5/8, SMAD2, and SMAD3 were analyzed by Western blotting. The immunoblot shown in the upper left panel is representative of two independent experiments. Cumulative results for quantitative densitometry of the experiments are shown in the remaining panels. Results represent the mean ± SEM of the experiments.

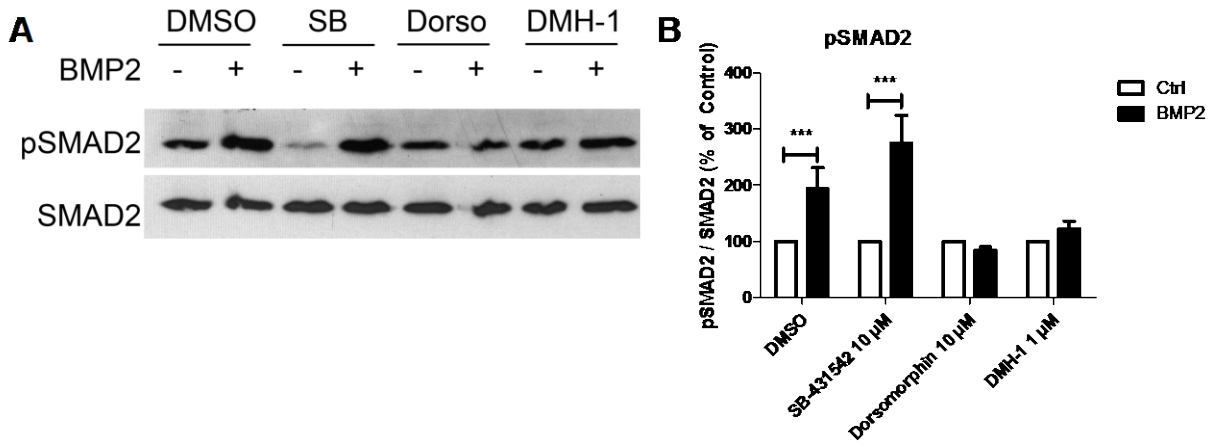

Figure 13. Dorsomorphin and DMH-1, but not SB-431542, blocks the BMP2-induced phosphorylation of SMAD2 in SKOV3.
SKOV3 cells were pretreated with vehicle control (DMSO), SB-431542 (10 mM), Dorsomorphin (10 µM), or DMH-1 (1 µM) for 1 h prior to the addition of vehicle control or 50 ng/mL BMP2 for 30 min. Phosphorylated and total SMAD2 were analyzed by Western blotting. The immunoblot shown in the left panel is representative of three independent experiments (A). Cumulative results for quantitative densitometry of the experiments are shown relative to the vehicle control for each group (B). Results represent the mean ± SEM of the experiments. Data were analyzed by one-way ANOVA followed by the Newman-Keuls multiple comparison test (**P < 0.01; ***P < 0.0001).

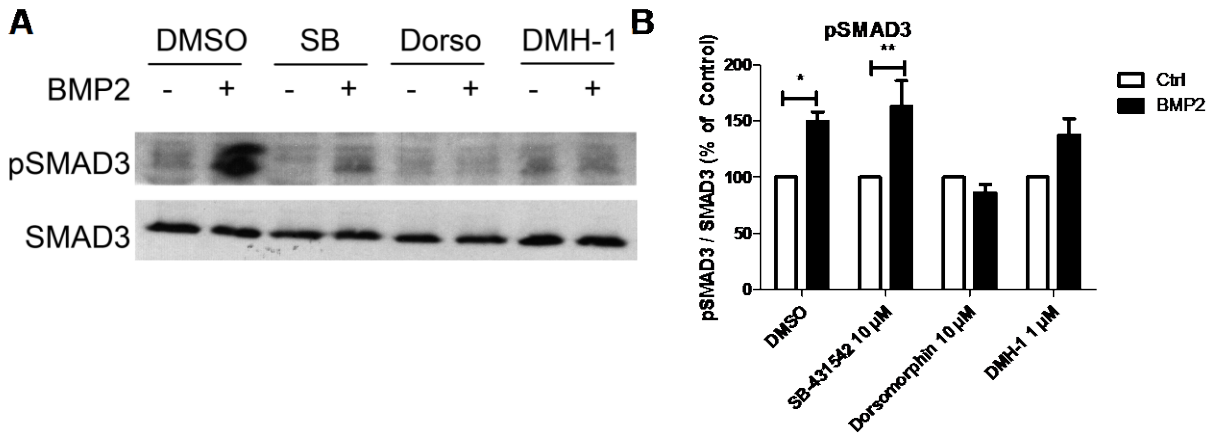

Figure 14. Dorsomorphin and DMH-1, but not SB-431542 blocks the BMP2-induced phosphorylation of SMAD3 in SKOV3.
SKOV3 cells were pretreated with vehicle control (DMSO), SB-431542 (10 µM), Dorsomorphin (10 µM), or DMH-1 (1 µM) for 1 h prior to the addition of vehicle control or 50 ng/mL BMP2 for 30 min. Phosphorylated and total SMAD3 were analyzed by Western blotting. The immunoblot shown in the left panel is representative of three independent experiments (A). Cumulative results for quantitative densitometry of the experiments are shown relative to DMSO control (B) or relative to the vehicle control for each group (C). Results represent the mean ± SEM of the experiments. Data were analyzed by one-way ANOVA followed by the Newman-Keuls multiple comparison test (*$P < 0.05$; **$P < 0.01$).

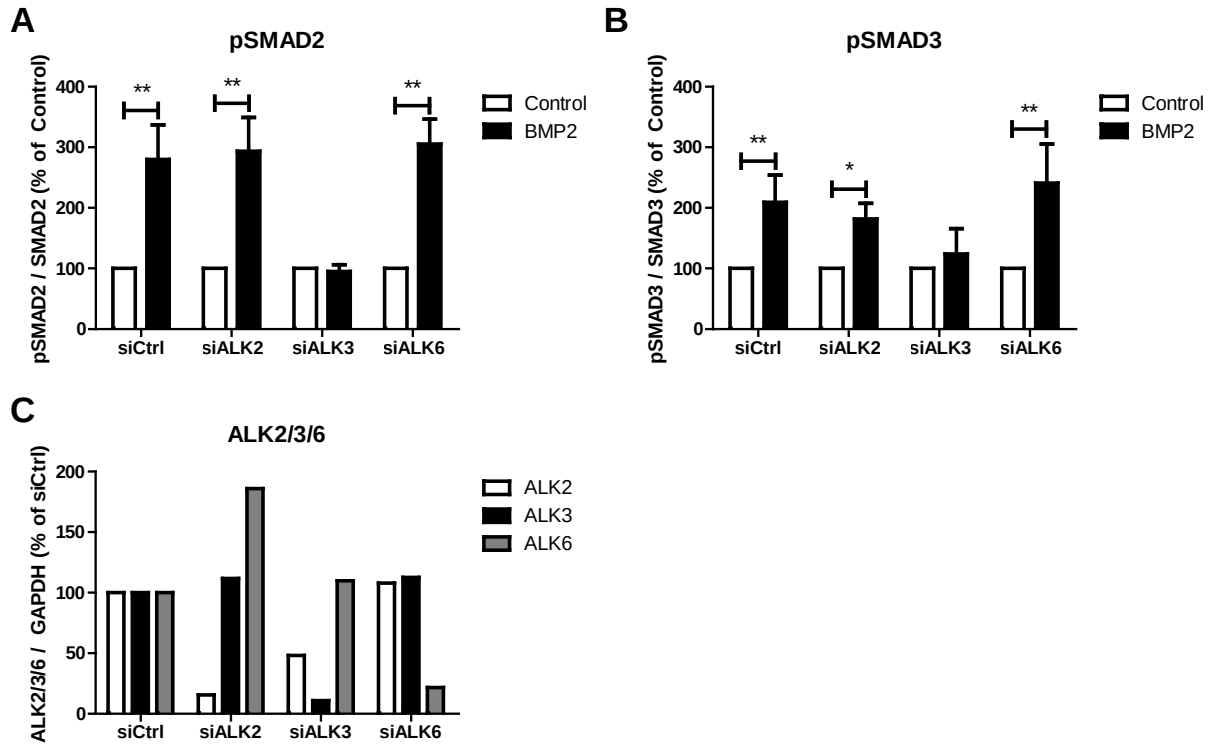

Figure 15. ALK3 mediates the BMP2-induced phosphorylation of SMAD2 and SMAD3 in SKOV3.

SKOV3 cells were transfected with 25 nM of control siRNA (siCtrl) or siRNA targeting ALK2 (siALK2), ALK3 (siALK3), or ALK6 (siALK6) for 48 h prior to the addition of vehicle control or 50 ng/mL BMP2 for 30 min. Phosphorylated and total SMAD2 (A) and SMAD3 (B) were analyzed by Western blotting. Cumulative results for quantitative densitometry of three experiments are shown. Results represent the mean ± SEM of the experiments. Data were analyzed by one-way ANOVA followed by the Newman-Keuls multiple comparison test (*$P < 0.05$; **$P < 0.01$). The efficiency of ALK knockdown was examined by TaqMan RT-qPCR with GAPDH as the reference gene using the comparative Cq ($2^{-\Delta\Delta Cq}$) method (C).

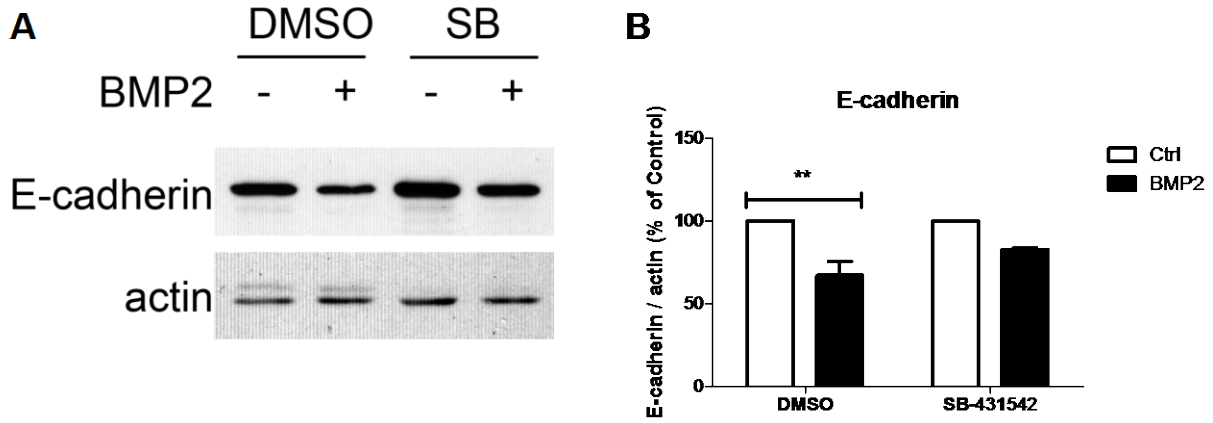

Figure 16. SB-431542 blocks the BMP2-induced down-regulation of E-cadherin protein levels in SKOV3.

SKOV3 cells were pretreated with vehicle control (DMSO) or SB-431542 (10 µM) for 1 h prior to the addition of vehicle control or 50 ng/mL BMP2 every 24 h for 72 h. E-cadherin protein levels were analyzed by Western blotting. The actin antibody was used as a loading control. The immunoblot shown in the left panel is representative of three independent experiments (A). Cumulative results for quantitative densitometry of the experiments are shown in the right panel (B). Results represent the mean ± SEM of the experiments. Data were analyzed by one-way ANOVA followed by the Newman-Keuls multiple comparison test (**P < 0.01).

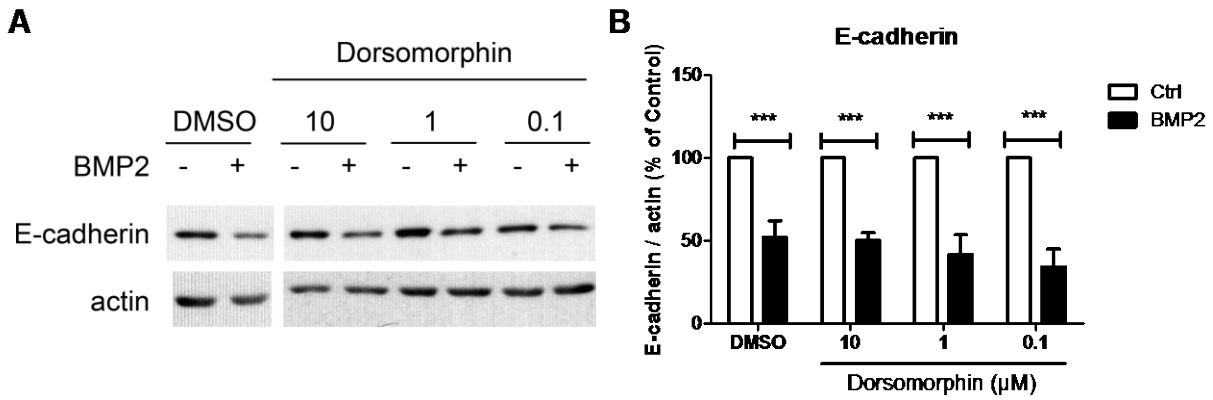

Figure 17. Dorsomorphin does not block the BMP2-induced down-regulation of E-cadherin protein levels in SKOV3.
SKOV3 cells were pretreated with vehicle control (DMSO) or decreasing doses of Dorsomorphin (10, 1, and 0.1 μM, as indicated) for 1 h prior to the addition of vehicle control or 50 ng/mL BMP2 every 24 h for 72 h. E-cadherin protein levels were analyzed by Western blotting. The actin antibody was used as a loading control. The immunoblot shown in the left panel is representative of three independent experiments (A). Cumulative results for quantitative densitometry of the experiments are shown relative to the vehicle control for each group (B). Results represent the mean ± SEM of the experiments. Data were analyzed by one-way ANOVA followed by the Newman-Keuls multiple comparison test (***$P < 0.0001$).

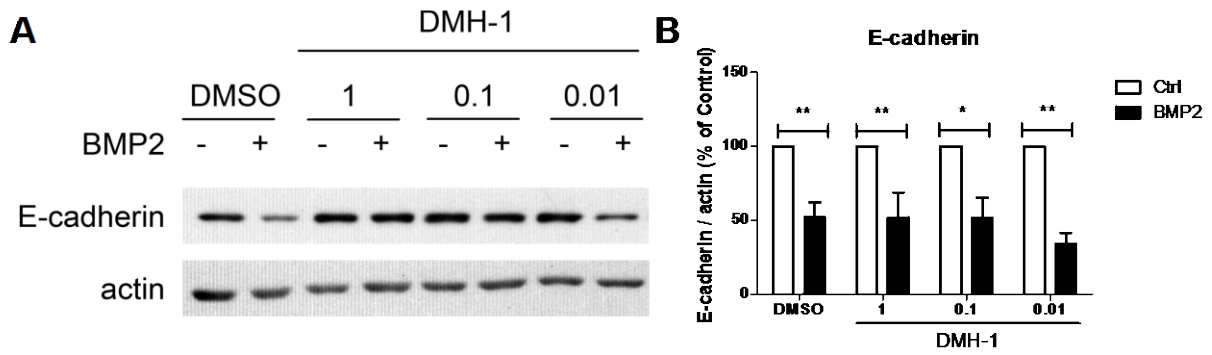

Figure 18. DMH-1 does not block the BMP2-induced down-regulation of E-cadherin protein levels in SKOV3.

SKOV3 cells were pretreated with vehicle control (DMSO) or decreasing doses of DMH-1 (1, 0.1, and 0.01 µM, as indicated) for 1 h prior to the addition of vehicle control or 50 ng/mL BMP2 every 24 h for 72 h. E-cadherin protein levels were analyzed by Western blotting. The actin antibody was used as a loading control. The immunoblot shown in the left panel is representative of three independent experiments (A). Cumulative results for quantitative densitometry of the experiments are shown relative to the vehicle control for each group (B). Results represent the mean ± SEM of the experiments. Data were analyzed by one-way ANOVA followed by the Newman-Keuls multiple comparison test (*P < 0.05; **P < 0.01).

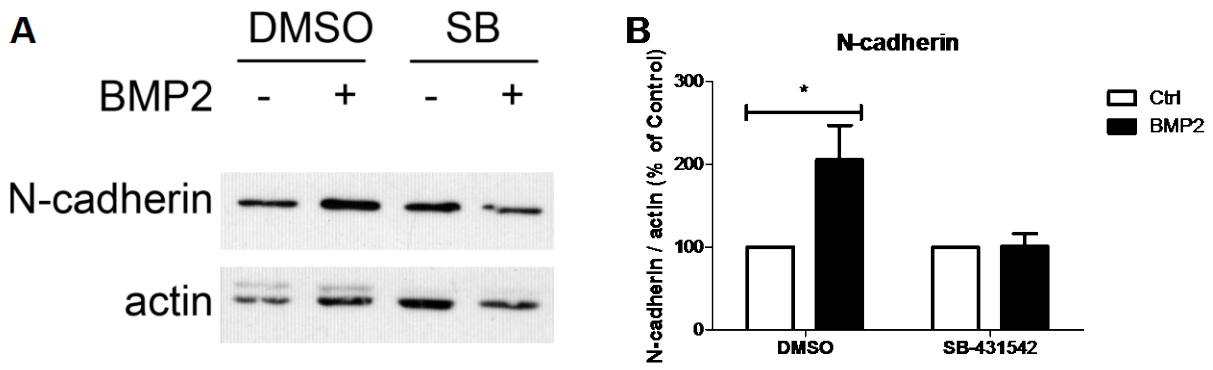

Figure 19. SB-431542 blocks the BMP2-induced up-regulation of N-cadherin protein levels in SKOV3.

SKOV3 cells were pretreated with vehicle control (DMSO) or SB-431542 (10 μM) for 1 h prior to the addition of vehicle control or 50 ng/mL BMP2 every 24 h for 72 h. E-cadherin protein levels were analyzed by Western blotting. The actin antibody was used as a loading control. The immunoblot shown in the left panel is representative of at least three independent experiments (A). Cumulative results for quantitative densitometry of the experiments are shown in the right panel (B). Results represent the mean ± SEM of the experiments. Data were analyzed by one-way ANOVA followed by the Newman-Keuls multiple comparison test (*$P < 0.05$).

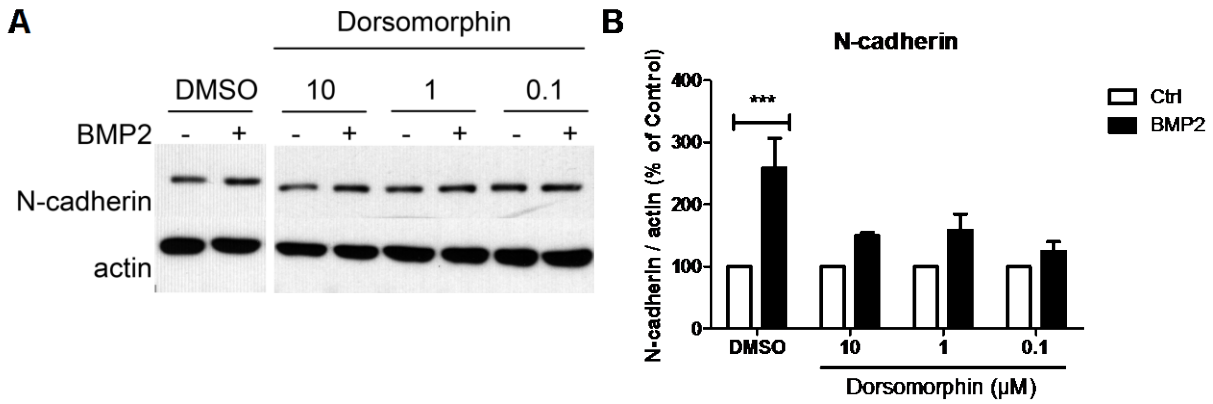

Figure 20. Dorsomorphin blocks the BMP2-induced up-regulation of N-cadherin protein levels in SKOV3.
SKOV3 cells were pretreated with vehicle control (DMSO) or decreasing doses of Dorsomorphin (10, 1, and 0.1 μM, as indicated) for 1 h prior to the addition of vehicle control or 50 ng/mL BMP2 every 24 h for 72 h. N-cadherin protein levels were analyzed by Western blotting. The actin antibody was used as a loading control. The immunoblot shown in the left panel is representative of three independent experiments (A). Cumulative results for quantitative densitometry of the experiments are shown relative to the vehicle control for each group (B). Results represent the mean ± SEM of the experiments. Data were analyzed by one-way ANOVA followed by the Newman-Keuls multiple comparison test (***P < 0.0001).

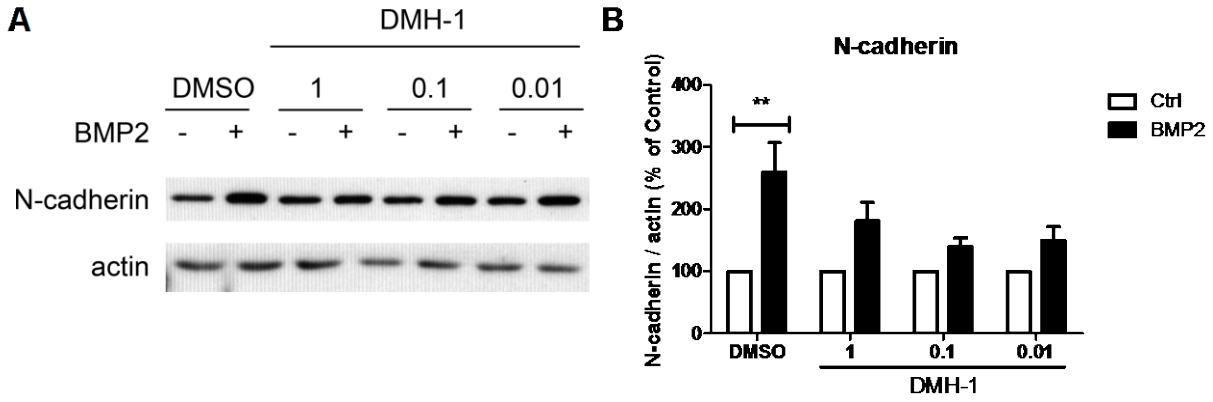

Figure 21. DMH-1 partially blocks the BMP2-induced up-regulation of N-cadherin protein levels in SKOV3.

SKOV3 cells were pretreated with vehicle control (DMSO) or decreasing doses of DMH-1 (1, 0.1, and 0.01 μM, as indicated) for 1 h prior to the addition of vehicle control or 50 ng/mL BMP2 every 24 h for 72 h. N-cadherin protein levels were analyzed by Western blotting. The actin antibody was used as a loading control. The immunoblot shown in the left panel is representative of three independent experiments (A). Cumulative results for quantitative densitometry of the experiments are shown relative to the vehicle control for each group (B). Results represent the mean ± SEM of the experiments. Data were analyzed by one-way ANOVA followed by the Newman-Keuls multiple comparison test (**P < 0.01).

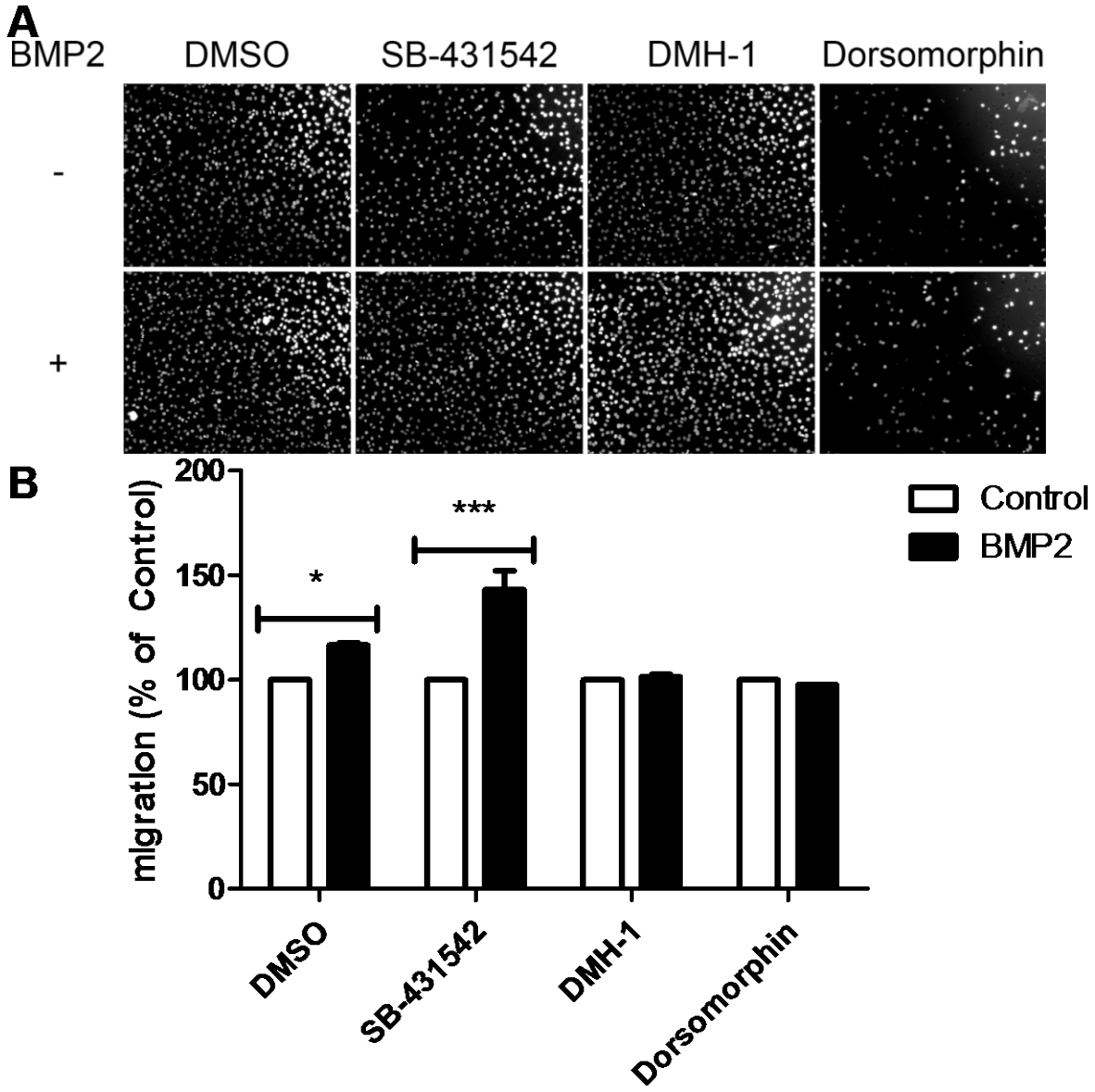

Figure 22. DMH-1 and Dorsomorphin, but not SB-431542, blocks the BMP2-induced migration in SKOV3.

SKOV3 cells were pretreated with vehicle control (DMSO), SB-431542 (10 µM), Dorsomorphin (10 µM), or DMH-1 (1 µM) for 1 h prior to the addition of vehicle control or 50 ng/mL BMP2 every 24 h for 72 h. After the final treatment, an equal number of cells were seeded into Transwell inserts and incubated for 24 h. Representative images of the migration assay of at least three independent experiments are shown (A). Cumulative quantitative results of the experiments are shown (B). Results represent the mean ± SEM of the experiments. Data were analyzed by one-way ANOVA followed by the Newman-Keuls multiple comparison test (*P < 0.05; ***P < 0.0001).

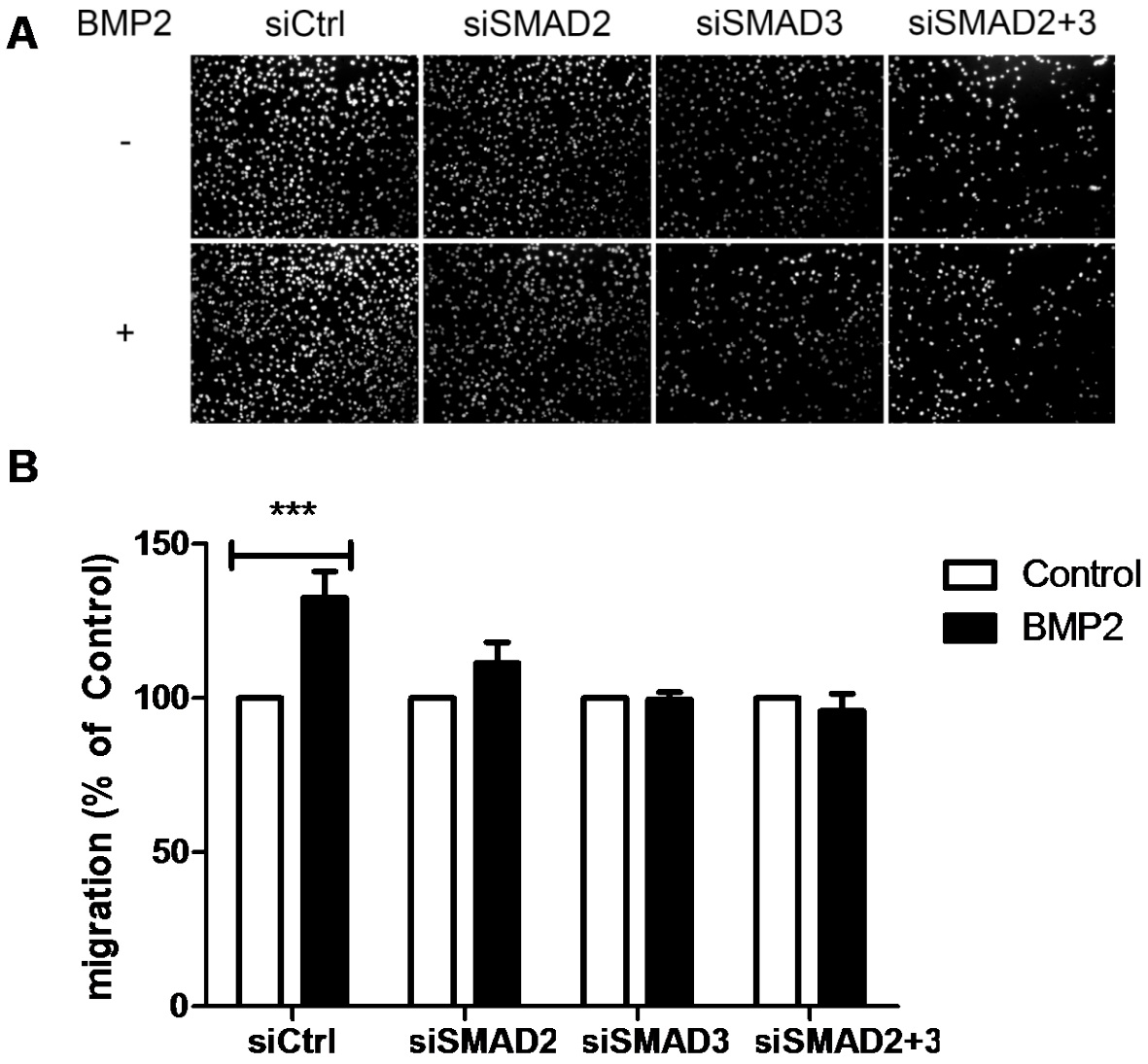

Figure 23. SMAD2 and SMAD3 are independently necessary for BMP2-induced migration in SKOV3.

SKOV3 cells were transfected with 50 nM control siRNA (siCtrl), 25 nM SMAD2 siRNA (siSMAD2), 50 nM SMAD3 siRNA (siSMAD3), or 25 nM SMAD2 siRNA in combination with 50 nM SMAD3 siRNA (siSMAD2+3) for 48 h. Cells were then treated with vehicle control or 50 ng/mL BMP2 every 24 h for 72 h. After the final treatment, an equal number of cells were seeded into Transwell inserts and incubated for 24 h. Representative images of the migration assay of four independent experiments are shown (A). Cumulative quantitative results of the experiments are shown (B). Results represent the mean ± SEM of the experiments. Data were analyzed by one-way ANOVA followed by the Newman-Keuls multiple comparison test (***$P<0.0001$).

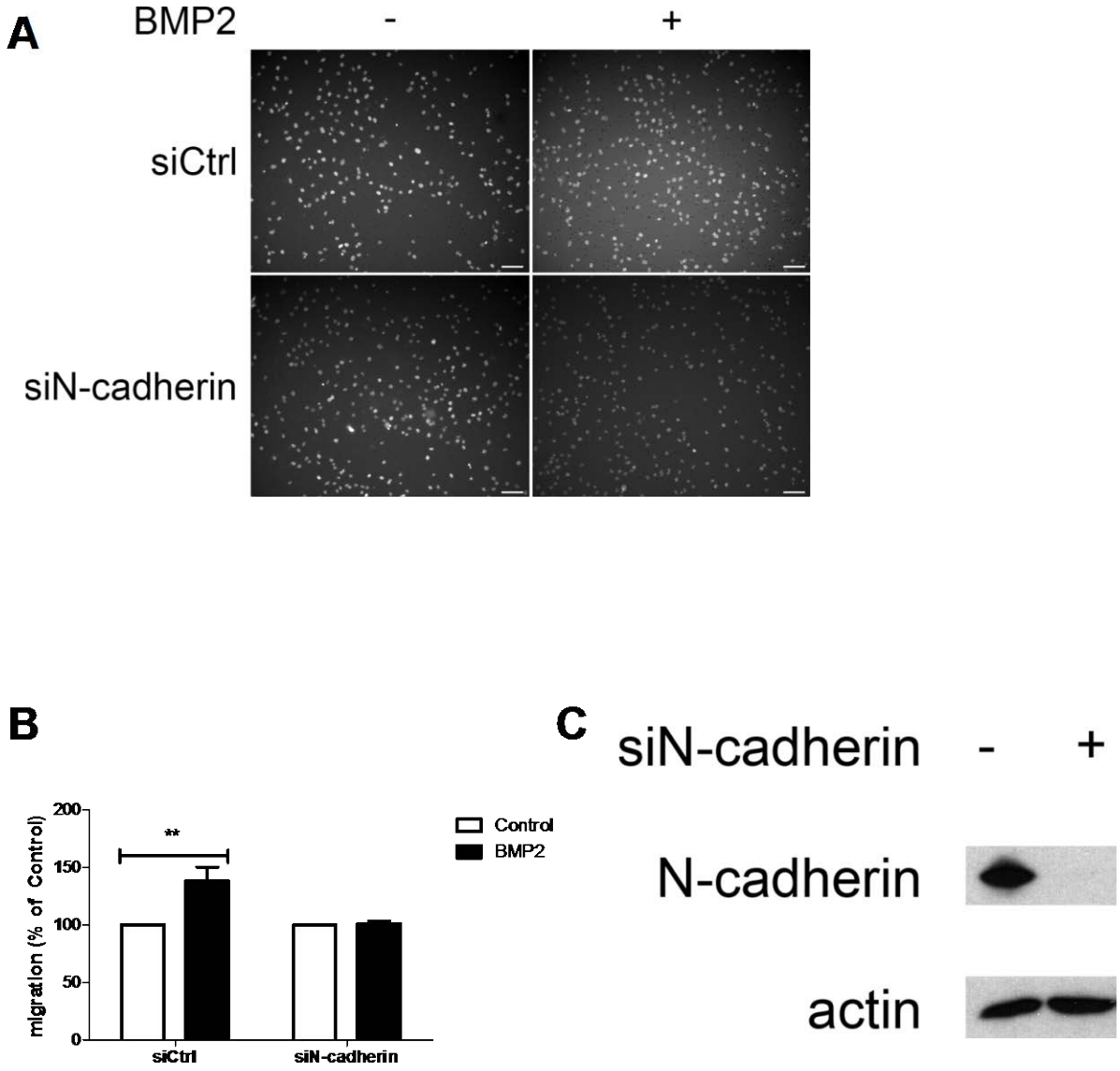

Figure 24. BMP2-induced migration is mediated by N-cadherin in SKOV3.
SKOV3 cells were transfected with 25 nM control siRNA (siCtrl) or 25 nM N-cadherin siRNA (siN-cadherin) for 48 h. Cells were then treated with vehicle control or 50 ng/mL BMP2 every 24 h for 72 h. After the final treatment, an equal number of cells were seeded into Transwell inserts and incubated for 24 h. Representative images of the migration assay of three independent experiments are shown (A). Cumulative quantitative results of the experiments are shown in the bottom-left panel (B). Results represent the mean ± SEM of the experiments. Data were analyzed by one-way ANOVA followed by the Newman-Keuls multiple comparison test (**P < 0.01). The efficiency of N-cadherin knockdown was analyzed by Western blotting with the actin antibody used as a loading control (C).

Chapter 4: Discussion

4.1 Non-canonical SMAD2/3 signaling

The traditional view of TGF-β signaling is that the type II receptor, being the primary point of contact for the numerous TGF-β superfamily of ligands, exhibits ligand promiscuity, whereas it is the type I receptor which is subsequently recruited and transphosphorylated by the type II receptor that confers specificity in SMAD activation. It is commonly regarded that the TGF-β superfamily of ligands signaling via the type I receptors ALK4, ALK5, or ALK7 activates the SMAD2/3 pathway, whereas the ligands signaling via the type I receptors ALK1, ALK2, ALK3, or ALK6 activates the SMAD1/5/8 pathway. Specifically, it is well known that although BMP family members can share type II receptors with the activin family, only binding of BMPs to the type II receptor recruits the BMP type I receptors ALK2, ALK3, or ALK6 for subsequent transphosphorylation and eventual activation of the SMAD1/5/8 pathway.

The present study joins a small but growing body of literature that challenges this traditional view and demonstrates non-canonical SMAD signaling mediated by type I receptors. Specifically, this study shows that BMP2 induces non-canonical SMAD2/3 signaling mediated by its canonical type I receptor, ALK3, in SKOV3 cells. BMP2 was similarly found to induce SMAD2/3 phosphorylation via ALK3 or ALK6 in a variety of cell types, notably in both established cancer cell lines and primary cancer cells [122-124].

Murakami et al. [124] suggests that non-canonical BMP2-stimulated SMAD2 signaling may be a consequence of high levels of ALK3 and ALK6, which corroborates my findings of low ALK6 mRNA expression and lack of SMAD2 signaling in both OVCAR5 and OVCAR8, but not SKOV3 cells. However, my data shows ALK6 is not necessary for non-canonical BMP2-stimulated SMAD2/3 signaling in SKOV3 cells, suggesting that ALK3 is the key

mediator in this system. Whether this reliance on ALK3 but not ALK6 for non-canonical BMP2 signaling is the exception or the norm in ovarian cancer cells remains unresolved at present.

Structural studies have shown the existence of heterodimeric type II receptor complexes, with the potential for both type II receptors to recruit one type I receptor each for a resulting receptor complex consisting of four different receptors [125]. Interestingly, Holtzhausen et al. [123] found that BMP2 unexpectedly complexed with the type II receptor typically used by TGF-β but not BMPs, TGFBR2, and mediates BMP2-induced SMAD2 phosphorylation while BMPR2 was found to mediate BMP2-induced SMAD3 phosphorylation. My data does not support the involvement of ALK4, ALK5, or ALK7 in non-canonical BMP2-stimulated SMAD2/3 phosphorylation, but the possibility remains for the use of the TGF-β type II receptor. Taken together, the mediation of SMAD2/3 signaling by ALK3 suggests that it is perhaps the type II receptor, rather than the type I receptor as commonly held, that confers SMAD signaling specificity.

4.2 N-cadherin is the primary cadherin driving BMP2-induced cell migration

E-cadherin downregulation and N-cadherin upregulation is described as the cadherin switch, and is a well-documented phenomenon during EMT that is associated with a motile and invasive phenotype (reviewed in [126-128]). Indeed, a BMP2-induced switch from E-cadherin to N-cadherin protein expression was observed in SKOV3 cells. While the decrease in E-cadherin mRNA levels at 24 h corresponding to the increase in E-cadherin protein levels at 72 h, N-cadherin mRNA levels remain unaffected at 24 h while N-cadherin protein levels were found to be increased at 72 h. Although my data suggests transcriptional regulation of cadherin switching is via Snail, Slug, and maybe ZEB1, reduced protein turnover may be contribute to

elevated N-cadherin protein levels. For instance, p120-catenin has been shown to interact with the cytoplasmic domain of classical cadherins to stabilize and prevent their degradation [129, 130]. Alternatively, it is possible that since N-cadherin protein levels were found to be significantly upregulated at 72 h, but not 48 h, mRNA measurements at 24 h are too early to show any appreciable N-cadherin mRNA induction.

It is commonly postulated that the loss of E-cadherin function is a necessary step for cancer cell migration. However, recent evidence indicates that this may not always be the case. Studies in breast cancer cell lines showed that overexpression of E-cadherin into N-cadherin expressing cells did not decrease N-cadherin levels or the migratory potential of the cells [131]. Conversely, overexpression of N-cadherin into breast cancer cell lines expressing E-cadherin did not decrease E-cadherin levels but increased the motility of the cells [131, 132]. In the present study, inhibition of BMP type I receptors blocked BMP2-induced SMAD2/3 signaling and N-cadherin up-regulation, but not the down-regulation of E-cadherin, yet still blocked the BMP2-induced cell migration in SKOV3 cells. Moreover, I show that siRNA-mediated knockdown of N-cadherin blocked the BMP2-induced migration in SKOV3 cells. Taken together, there is evidence demonstrating that N-cadherin has a dominant and critical role over E-cadherin in promoting cellular migration in ovarian cancer cells.

4.3 Inhibitors, crosstalk, and off-target effects

Interestingly, I observed that SB-431542 mediated inhibition of TGF-β type I receptors blocked both the down-regulation of E-cadherin and up-regulation of N-cadherin, but not SMAD2/3 signaling, and failed to block the BMP2-induced cell migration in SKOV3 cells. These results suggest that in this case, impairment of SMAD2/3 signalling is required to block

the BMP2-induced cell migration in SKOV3 cells. Furthermore, as TGF-β type I receptors are known to mediate SMAD1/5/8 signaling, these findings may suggest the crosstalk of canonical SMAD1/5/8 signaling with non-canonical SMAD2/3 signaling, whereby the SMAD1/5/8 pathway may affect the SMAD2/3 pathway, and vice versa. A recent study found that canonical SMAD1/5/8-responsive genes were coincidentally expressed with canonical SMAD2/3-responsive genes in breast and liver tumour specimens [123]. Moreover, SB-431542 has been reported to have off-target inhibition of other protein kinases, some of which are known members of the BMP SMAD-independent pathways [133]. Collectively, this supports the possibility of crosstalk in this system.

Likewise, the significant decrease in basal cell migration upon Dorsomorphin but not DMH-1 pre-treatment may also suggest the presence of off-target effects. Indeed, specificity tests of Dorsomorphin at concentrations sufficient to inhibit BMP signaling have demonstrated off-target inhibition of numerous other protein kinases [133-135]. It is therefore not unreasonable to expect that some of these off-target effects of Dorsomorphin, whether known or unknown, contribute to the observed significant decrease in basal migration that cannot be rescued by BMP2.

4.4 BMP2 in ovarian cancer

BMP2 plays an important physiological role in various tissues, while at the same time has been detected in various tumour tissues. Moreover, the effect of BMP2 varies depending on the type of cancer, and has been found to vary within a cancer type. Indeed, studies by Kiyozuka et al. [136] and Le Page et al. [105] have detected the expression of BMP2 in primary ovarian cancer tissues, and further studies by Le Page et al. [106] found an inverse correlation between

BMP2 expression in ovarian tumour tissue and survival in advanced stage patients. Conversely, Soda et al. [94] reported that 2 of 15 ovarian cancer tissue specimens demonstrated exogenous BMP2-mediated growth inhibition, while Ma et al. [107] found a correlation in advanced stage patients between positive BMP2 expression in ovarian tumour tissue with increased survival relative to patients with negative expression for BMP2. However, roughly one-third of the high-stage ovarian cancer specimens evaluated by Le Page et al. [106] were of the serous subtype, with the remaining two-thirds comprising of the endometrioid and clear cell subtypes, while the subtypes of the ovarian cancer specimens evaluated by Soda et al. [94] and Ma et al. [107] were not specified and the number of specimens evaluated were limited. Thus, these conflicting reports may be a function of differential representation of major ovarian cancer subtypes, each with their associated molecular features and clinical behaviours (reviewed in [6, 137, 138]).

The present study has demonstrated the migratory effects of BMP2 in the SKOV3 ovarian cancer cell line, which is in line with the observed inverse correlation between BMP2 and survival in patients [106]. Moreover, this study uncovers the molecular basis driving this phenomenon in SKOV3. Additionally, I demonstrated that this behaviour is not universal throughout the tested ovarian cancer cell lines, as BMP2 was not found to be pro-migratory in OVCAR8 cells, and only marginally pro-migratory in OVCAR5 cells. Similar to the conflicting reports of Le Page et al. [106] and Ma et al. [107], this discrepancy may be due to subtype-specific responses. Both OVCAR5 and OVCAR8 are thought to belong to the high-grade serous subtype, as molecular characteristics of these cells as well the histology of xenografts derived from these cell lines were consistent with high-grade serous [139, 140]. In contrast, SKOV3 has extensively used as a model for high-grade serous ovarian cancer, but recent evidence has demonstrated the unlikelihood of SKOV3 being high-grade serous and questions its use as such

[140-142]. Instead, SKOV3 is now proposed to belong to the clear cell or endometrioid subtype, which has a high degree of overlap in their molecular characteristics (reviewed in [137, 138]), based on its molecular profile and histology when grown as a xenograft [140-142]. It is therefore possible that the BMP2-induced migratory effect found in SKOV3 is applicable to the clear cell or endometrioid ovarian tumours, but not in high-grade serous ovarian tumours.

However, all of the experiments presented in this study were performed with cell lines cultured in an artificial environment. This *in vitro* system may not accurately reflect the peritoneal microenvironment of ovarian tumours *in vivo*. However, this *in vitro* model was readily accessible and allowed us usage of both pharmacological inhibitors and siRNA to examine specific signaling pathways or molecules of interest to elucidate the molecular mechanisms that mediate ovarian cancer migration. Therefore, a three-dimensional cell culture system that mimics *in vivo* conditions and use of a mouse xenograft model can provide additional insights into ovarian tumour biology.

Additionally, the cell lines used were established many decades ago and their origins are poorly defined and there has been much debate about their validity in representing specific ovarian cancer subtypes (e.g. SKOV3). Recent advances in the histopathological diagnosis of ovarian cancer have led to a simplified and accurate classification of tumours into the five major subtypes. Therefore, the field would benefit from the establishment of a new panel of cell lines with representative histological and molecular characteristics that reflect the various originating subtypes. A better understanding of subtype-dependent molecular pathways will provide insight into therapeutic avenues such as personalized medicine and will have implications in the control of ovarian cancer.

4.5 Clinical relevance of non-canonical SMAD2/3 signaling

In this study, I demonstrated the importance of non-canonical SMAD2/3 signaling in mediating the BMP2-induced cell migration response in ovarian cancer cells. Indeed, cell lines that show a lack of BMP2-induced SMAD2/3 signaling, as in seen in OVCAR8 cells, which repressed SMAD3 phosphorylation while not affecting SMAD2 phosphorylation levels, failed to exhibit BMP2-induced morphological changes and increases in its migratory potential. Likewise, OVCAR5 cells, which may increase SMAD3 phosphorylation levels but not for SMAD2 in response to BMP2, exhibited a marginal increase in cell migration. On the other hand, SKOV3 cells, which exhibited non-canonical induction of SMAD2 and SMAD3 phosphorylation upon BMP2 treatment presented with EMT-like morphological changes and increases in its migratory potential, the latter of which was found to be blocked upon SMAD2/3 siRNA-mediated gene silencing. Other groups have shown that silencing of SMAD3 in pancreatic and breast cancer cell lines that exhibit non-canonical BMP2 signaling blocked BMP2-mediated cell invasion [123].

Given the identification of several distinct histotypes of ovarian cancer (reviewed in [7, 9, 137, 143]) and the genetic variation within histotypes [144], effective treatment options should be personalized based on the signaling pathways driving cancer progression. I therefore propose that by evaluating the signaling output upon exogenous BMP2 treatment, notably the quick SMAD2/3 phosphorylation response, cells and tumours with responses similar to that of SKOV3 can be rapidly identified.

Chapter 5: Conclusion and Future Studies

5.1 Future studies

5.1.1 Determination of the BMP2 response across various ovarian cancer histotypes

My data indicated variation in the BMP2 response across the three cell lines examined. Based on the assumed histotype of the cell lines used, my data demonstrates the possibility of histotype-specific responses to BMP2. Likewise, previous studies with primary ovarian tumour tissues have demonstrated variation in the BMP2 response across different tumours, but it is unclear if this variation is attributed to differences in histotypes. Future studies should evaluate tumour samples and additional cell lines representative of the major histological subtypes of ovarian cancer to determine if the BMP2 response is indeed histotype-specific. Tissue microarrays should be used to determine if BMP2 is differentially expressed across the major ovarian cancer subtypes, and if correlations between BMP2 expression and survival, stage, or grade exists within particular subtypes.

5.1.2 Identification of the TGF-β type II receptor that mediates BMP2-induced non-canonical SMAD signaling

My data revealed that non-canonical BMP2-induced SMAD2/3 phosphorylation is mediated by the TGF-β type I receptor ALK3 in SKOV3 cells. In canonical TGF-β signaling, TGF-β would directly bind to TGFBR2 homodimers. Likewise, in canonical BMP signaling, BMPs would directly bind to dimerized type II receptors of ACVR2A, ACVR2B, or BMPR2, but not TGFBR2. Heterodimeric type II receptor complexes have been recently described [125], and together with reports of BMP2 complexing with TGFBR2 to facilitate non-canonical BMP2-induced SMAD2 phosphorylation in breast cancer cell lines [123], raises the possibility of a

similar receptor arrangement facilitating non-canonical SMAD signaling in ovarian cancer. Future studies should identify (1) the type II receptor(s) involved in non-canonical BMP2-induced SMAD2/3 signaling in ovarian cancer, and (2) the role, if any, of heterodimeric type II receptor arrangements mediating this signaling pathway.

5.1.3 Determination of the involvement of SMAD-independent pathways for BMP2-stimulated ovarian cancer cell migration

Several SMAD-independent pathways downstream of TGF-β/BMP receptors have been identified, including the MAPK/ERK, p38 MAPK, JNK, and PI3K/Akt pathways, among others (reviewed in [145, 146]). Convergence and crosstalk of the SMAD-dependent and SMAD-independent pathways have been described, whereby non-SMAD pathway members have been shown to activate SMADs, and vice versa (reviewed in [145]). The present study has shown a clear role of SMAD2/3 in BMP2-induced cell migration, but the involvement of non-SMAD pathways in facilitating this response via crosstalk remains unclear. Future studies should identify (1) SMAD-independent pathways activated in response to BMP2 stimulation, and (2) the link, if any, between the pathways identified in (1) and SMAD-dependent signaling in mediating BMP2-induced ovarian cancer cell migration.

5.2 Conclusion

The findings in this study have provided evidence of the involvement of non-traditional SMAD-activation via BMP2 as a mediator of ovarian cancer cell migration. To better understand the molecular mechanisms involved in BMP2-induced ovarian cancer cell migration, this work focused on identifying the downstream signaling upon ALK activation. My results

suggest that although both canonical SMAD1/5/8 and non-canonical SMAD2/3 signaling is induced by BMP2, it is SMAD2 and SMAD3 that are the important driving forces in the BMP2-induced E-cadherin to N-cadherin switch, which in turn mediates the BMP2-induced migration in ovarian cancer cells. Moreover, this study suggests that the pro-migratory role of N-cadherin may be dominant over the tumour-suppressive role of E-cadherin, and the BMP2 response in ovarian cancer may be subtype-dependent.

These findings continue to deepen our understanding of the differential roles of BMP2 in ovarian cancer progression, and may play a role in the development of new therapeutics and improved screening strategies.

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