THE ROLE OF BONE MORPHOGENETIC PROTEIN 4 SIGNALLING IN HUMAN OVARIAN CANCER CELL BEHAVIOUR

by

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ABSTRACT

Ovarian cancer (OvCa) is the most lethal of the gynecological malignancies, and its mechanisms of carcinogenesis are poorly understood. Most OvCas are thought to arise from the ovarian surface epithelium (OSE). An autocrine bone morphogenetic protein 4 (BMP4) signalling pathway in primary human normal OSE and OvCa cells has been identified in our laboratory, and this study investigated the behavioural consequences of BMP4 signalling in OSE and OvCa cells. BMP4 treatment of OvCa cells produced morphological alterations and increased cellular adhesion, motility and invasion. The BMP4 inhibitor Noggin blocked the BMP4-induced phenotype, and decreased autocrine BMP4-mediated OvCa cell motility and adherence. Exogenous BMP4 upregulated the epithelial-mesenchymal transition (EMT) markers Snail and Slug while E-cadherin was downregulated, and the network of alpha smooth muscle actin (αSMA) changed to resemble a mesenchymal cell. Levels of activated Rho-GTPases in OvCa cells were changed, suggesting that the BMP4-induced cellular behaviours are likely mediated through the activation of these molecules. Treatment of OSE cells with BMP4 or Noggin failed to alter cell motility, providing evidence that OSE and OvCa cells possess distinct capacities to respond to BMP4. The BMP4-induced changes in morphology and motility were shown to be Smad-dependent through the RNA-mediated targeting of Smad expression. These studies suggest a link between autocrine BMP signalling mediated through Smad-dependent signalling, the Rho-GTPases, and Snail/Slug-induced EMT that may collectively contribute to aggressive OvCa behaviour.

Transforming growth factor beta (TGFß) and Activin signalling pathways were also found to be intact in OSE and OvCa cells, where exogenous ligand stimulated Smad phosphorylation and target gene upregulation. BMP6, TGFß1 and ActivinA also induced morphologic changes characterized by cell spreading. Interestingly, TGFß1 and ActivinA induced inhibition of OvCa growth and motility, while BMP4 and BMP6 increased cellular motility, having no proliferative effect. TGFß and Activin inhibitors increased cellular growth and motility to above control levels, indicating that autocrine TGFß/Activin signalling also exerts control over OvCa behaviours. Taken together, these results suggest that a balance exists between BMP and TGFß/Activin signalling within OSE and/or OvCa cells, and that this balance is altered to favor BMP signalling during OvCa metastatic progression.

LIST OF ABBREVIATIONS AND SYMBOLS USED

ABP	actin binding protein
Ad	adenovirus
ALK	Activin like kinase
α	alpha
αSMA	alpha smooth muscle actin
ARIP	Activin receptor interacting protein
ActRII	Activin receptor type II
BAMBI	BMP and Activin membrane bound inhibitor
ß	beta
bFGF	basic fibroblast growth factor
bHLH	basic helix-loop-helix
BISC	BMP-induced signalling complex
BMP	bone morphogenetic protein
BMPR	bone morphogenetic protein receptor
BRAF	v-raf-murine sarcoma viral oncogene homolog B1
BRCA	breast cancer
BRE	BMP response element
°C	degrees Celsius
CA125	cancer antigen 125
cAMP	cyclic AMP
CCP	clathrin-coated pit
cDNA	complementary DNA

CED	Camurati-Engelmann Disease
CLDN3	Claudin 3
cm^2	centimetre square
COX2	cyclooxygenase 2
CS	Cowden-like syndrome
CUTL1	cut-like 1
CXCR4	chemokine (C-X-C) motif receptor 4
CXCL12	chemokine (C-X-C) motif ligand 12
DAPK	death associated protein kinase
DMEM	Dulbecco's modified Eagles medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DPM	disintegrations per minute
DRM	detergent-resistant membrane fraction
DTT	dithiothreitol
ECL	enhanced chemiluminescence
erbB2/neu/HER2	v-erb-2 erythroblastic leukemia viral oncogene homolog 2/neuro/glioblastoma derived oncogene homolog
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EGTA	ethylene glycol tetraacetic acid
EMT	epithelial-mesenchymal transition

ER	estrogen receptor
ERK	extracellular signal related kinase
F-actin	filamentous actin
FAP	focal adhesion protein
FAS	tumor necrosis factor superfamily member 6
FBS	fetal bovine serum
FIGO	International federation of gynecology and obstetrics
FLRG	Follistatin related gene
FOP	fibrodysplasia ossificans progressiva
FSH	follicle stimulating hormone
GAPDH	glyceraldehyde phosphate dehydrogenase
GFP	green fluorescent protein
GnRH	gonadotropin releasing hormone
GRK2	G-coupled receptor kinase 2
GS	glycine-serine motif
GTPase	guanosine triphosphatase
h	hour
HA	hemagglutinin
HEPES	(4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid)
HGF	hepatocyte growth factor
HRP	horseradish peroxidase
Id	inhibitor of DNA binding/inhibitor of differentiation
IF	intermediate filament

IgG	immunoglobulin G
IGFII	insulin growth factor II
IL	interleukin
IP	intraperitoneal
IV	intravenous
JNK	Jun-N terminal kinase
JPS	juvenile polyposis syndrome
kDa	kilo Dalton
KGF	keratinocyte growth factor
kitL	kit ligand
KRAS	v-ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LAP	latency associated peptide
LH	luteinizing hormone
LIMK	LIM kinase
LOH	loss of heterozygosity
LPA	lysophosphatidic acid
LSA	lipid associated sialic acid
LTBP	latent TGFß binding protein
m	minute
MAPK	mitogen activated protein kinase
M-CSF	macrophage colony stimulating factor
MEN1	multiple endocrine neoplasia 1
MET	mesenchymal to epithelial transition

MFS	Marfan syndrome
$MgCl_2$	magnesium chloride
MH1	mad-homology 1
MISIIR	müllerian inhibiting substance receptor type II
mL	millilitre
mm	millimetre
mM	millimolar
MMP	matrix metalloprotease
MOI	multiplicity of infection
mRNA	messenger RNA
MUC1	mucin 1
M199	medium 199
N	normal
NaCl	sodium chloride
NaF	sodium fluoride
NaOH	sodium hydroxide
ng	nanogram
nm	nanometre
NT	non transduced
O/N	overnight
OSE	ovarian surface epithelium
OvCa	ovarian cancer
PAI-1	plasminogen activator inhibitor 1

PBS	phosphate-buffered saline
pcofilin	phospho-cofilin
PDGF	platelet derived growth factor
PDP	pyruvate dehydrogenase phosphatase
PFA	paraformaldehyde
PFC	preformed receptor complex
PI3K	phosphoinositide-3 kinase
PKA	protein kinase A
PKB	protein kinase B
pM	picomolar
PMSF	phenylmethanesulphonylfluoride
PPH	primary pulmonary hypertension
PP2Cα	protein phosphatase 2C alpha
PR	progesterone receptor
pRb	tumor protein retinoblastoma
P/S	penicillin/streptomycin
PTEN	phosphatase and tensin homolog
p53	tumor protein 53
p300/CBP	E1A binding protein p300/CREB binding protein
/	per
%	percent
QPCR	quantitative reverse-transcriptase polymerase chain reaction
RAB25	Ras oncogene family 25

RNA	ribonucleic acid
ROCK	Rho kinase
rpm	revolutions per minute
R-smad	receptor activated smad
RT	room temperature
s	second
SARA	Smad anchor for receptor activation
SC	subcutaneous
SCP	small C-terminal phosphatase
SHIP	Src homology 2 domain-containing 5' inositol phosphatase
shRNA	small hairpin RNA
Smurf	smad ubiquitination regulatory factor
STAT3	signal transducer and activator of transcription 3
SV40	Simian virus 40
TAK1	TGFß-activated kinase 1
TBST	Tris buffered saline with 0.05% Tween 20
TßRII	transforming growth factor beta type II receptor
TGFß	transforming growth factor beta
TLP	Trap-1-like protein
TNFα	tumor necrosis factor alpha
Tob/BTG	transducer of erbB2/B-cell translocation gene
Trb3	Tribbles-like protein 3
U	units

μg	microgram
μL	microlitre
μm	micrometre
uPA	urokinase-like plasminogen activator
uPAR	urokinase-like plasminogen activator receptor
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor

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I have learned many things in grad school, including: haemacytometer are made of glass and will break when dropped, nitrocellulose membrane is flammable, never sneeze on your cultures, or they will get contaminated, it's amazing what kind of artwork you can make out of pipette tips and a Bunsen burner, coffee and Bailey's do go very well together, and most importantly, when everything else fails...you could always talk about knitting!

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CHAPTER 1 - INTRODUCTION

PREAMBLE

This body of work investigates the behavioural implications of BMP signalling in primary human OvCa cells. My hypothesis is that BMP4 will promote aggressive cellular behaviours of primary human OvCa cells *in vitro*. Assessments of adhesion, motility, invasion and proliferation in response to exogenous BMP4 were performed to evaluate cellular responses. Furthermore, the contribution of autocrine BMP signalling in OvCa cell behaviours was revealed through the use of the BMP inhibitor Noggin. Effects on motility obtained in response to BMP4 in primary OvCa cells were then compared to those of primary normal OSE cells, and significant differences between how these two cell types respond were observed. Intracellular mediators responsible for these BMP4-induced effects were then examined and identified. This study evolved to investigate the autocrine contributions of other members of the TGFß superfamily of ligands, such as BMP6, TGFß1 and Activin A through the use of exogenous ligands with the combination of specific inhibitors. The comparison of the behavioural responses obtained through autocrine signalling exposed antagonistic effects between TGFß/Activin signalling and BMP signalling in OvCa cells. Based on the different *in vitro* contributions of TGFß superfamily signalling members on OvCa cell biology, a model of OvCa cell tumor metastatic progression was proposed.

1.1 Ovarian Cancer

OvCa is the most lethal of the gynecological malignancies (Tortolero-Luna et al., 1994), and the fourth leading cause of death in women in North America. According to the Canadian Cancer Society, there will be in 2007 an estimated 2400 new diagnoses and 1700 deaths due to this disease in Canada. Although women only have a 1 in 70 probability of developing this disease over their lifetime, the 5-year survival rate for women suffering from OvCa is 38% (2007). This poor prognosis is primarily due to the fact that most OvCas (~ 80%) are detected at advanced stages, when the cancer has metastasized to other organs. When OvCa is found at earlier stages (~ 20% of cases), the 5-year survival rate increases to 94%. The disease often develops and progresses without any overt symptoms, and when symptoms do occur, they are often non-specific (Williams et al., 2007). Symptoms include gastrointestinal disturbances, nausea, cramps, headaches, or bloating; these symptoms could be attributed to benign causes or associated with a number of disease processes other than OvCa (Goff et al., 2007). However, Goff *et al* recently developed an OvCa symptom index that lists symptoms that were associated significantly with OvCa: pelvic/abdominal pain, urinary frequency/urgency, increased abdominal size/bloating, and difficulty eating/feeling full (Goff et al., 2007). These symptoms were determined to be cause for concern and correlative with the development of OvCa when they were present for less than 1 year, and occurred more than 12 days per month; therefore this index could become a useful tool in the early detection of women suffering from OvCa (Goff et al., 2007). Metastatic disease progression is often characterized by the formation of ascites fluid within the peritoneal cavity, which causes a visible distention of the abdominal cavity and is for many women,

the first physical sign of disease progression. Although a number of effective therapies exist for the first-line treatment of OvCa (discussed below), for reasons that are not yet understood, many patients with OvCa will have recurrent disease which is resistant to conventional chemotherapeutics (Aletti et al., 2007; Chien et al., 2007). Screening tests to detect the disease at early stages are currently unavailable but are actively being sought by OvCa researchers (Williams et al., 2007).

1.1.1 Types Of Ovarian Cancer

Based on the cells of origin, OvCas can be classified into three main groups: epithelial ovarian cancer, which is the most common type of ovarian malignancy and is thought to arise from the OSE (Auersperg et al., 2001), accounting for ~80% of OvCas; stromal cell tumors, which are tumors arising from the stromal cells or connective tissue of the ovary and comprise 10-15% of OvCas; and germ cell tumors which comprise 5-10% of OvCas (Williams et al., 2007). Epithelial ovarian cancer, which is the most common and most widely studied type of ovarian cancer, will be the focus of this thesis and will be referred to from this point forward as OvCa.

1.1.2 Epithelial Ovarian Cancer (OvCa) Pathology

OvCas are heterogeneous in nature, and are further subdivided into 5 major categories based on tumor histology resembling the morphology of different epithelia of the female reproductive tract. Serous tumors are the most common type, comprising ~ 40% of all OvCa tumors, and resemble the epithelium of the fallopian tube; endometrioid OvCa comprises ~ 20% of tumors and resembles endometrial cells; clear cell which comprises 6-10% of tumors and also resembles endometrial cells; mucinous comprises 1-4% of tumors and resembles the endocervical epithelium; and 15% of OvCas are

undifferentiated carcinomas, which do not resemble any of the previous subtypes or any of the gynecologic tissues. The remaining subtypes (~15%) are generally borderline tumors that do not appear cancerous and they are characterized as having low malignant potential, showing serous or mucinous histology and tending to occur in younger women (Scully, 1995; Williams et al., 2007). The extent of spread (staging) and aggressiveness (grading) of OvCas is examined by surgical removal of the tumor, along with biopsies of normal and suspicious areas throughout the peritoneal cavity followed by pathological examination of the tissues obtained from these biopsies. Gynecologic tumors are usually staged according to the guidelines issued by the International Federation of Gynecology and Obstetrics (FIGO) (Benedet et al., 2000). Stage I consists of tumors confined to the ovaries; stage Ia represents the tumor confined to one ovary, stage Ib represents tumors present in both ovaries, while stage Ic consists of tumors on one or both ovaries with the presence of ascites fluid or malignant cells in the peritoneal fluid. Stage II consists of tumors on one or both ovaries with pelvic extension; stage IIa represents the cancer extending or metastases present on the uterus and/or fallopian tubes, stage IIb represents the cancer extending to other pelvic tissues, and stage IIc represents the cancer extending to reproductive tissues or other pelvic tissues with the presence of ascites or malignant cells in the peritoneal fluid. Stage III consists of tumors on one or both ovaries with microscopically confirmed metastasis outside the pelvic area; stage IIIa represents seeding of the cancer cells on abdominal peritoneal surfaces with negative lymph nodes, stage IIIb represents confirmed cancer cell implants on peritoneal surfaces that are less than 2 cm in diameter with negative lymph nodes, while stage IIIc represents peritoneal cancer cell implants over 2 cm in diameter, with positive peritoneal or inguinal lymph

nodes. Stage IV consists of tumors present on both ovaries with distant metastases, including parenchymal liver and pleural metastases (Benedet et al., 2000; Williams et al., 2007). Tumors can be further subclassified by grading, which will reveal the microscopic features of the malignant cells and describe how they differ from normal cells, and is also a measure the aggressiveness of the tumor (Aletti et al., 2007; Williams et al., 2007). Gx denotes that the grade cannot be assessed, G1 denotes that the cells are well differentiated, G2, the cells are moderately differentiated, and G3, the cells are poorly differentiated. The prognosis of a patient will decrease with an increasing grade, as the more poorly differentiated cells tend to be more aggressive (Aletti et al., 2007; Williams et al., 2007).

1.1.3 OvCa Risk Factors

A number of environmental, hormonal and hereditary factors have been associated with the increased risk of developing OvCa. The median age for women suffering from OvCa is 60 (Cannistra, 2004). Reports on the effect of diet and exposure to carcinogens such as radiation, asbestos and talc on OvCa risk are conflicting (Aletti et al., 2007; Auersperg et al., 2001; Williams et al., 2007). However, epidemiological studies have shown that hormonal factors such as pregnancy and oral contraceptive use will decrease a woman's risk of developing OvCa (Risch, 1998), while nulliparity, early menarche and late menopause will increase the risk of OvCa development. These risk factors stem from two theories of OvCa etiology proposed by Fathalla *et al* in 1971, and Stadel *et al* in 1975, where the risk of developing OvCa increases with the number of ovulatory events. The incessant ovulation theory proposed by Fathalla states that the frequent cycles of proliferation and cellular repair that the OSE experiences during

ovulation causes these cells to accumulate genetic mutations that could predispose them to malignant transformation (Fathalla, 1971). In the same manner, the gonadotropin theory proposed by Stadel postulates that the cyclical surge in gonadotropins responsible for initiating ovulatory events stimulates cellular proliferation and induces cellular transformation (Stadel, 1975). Both of these theories suggest a link between chronic ovulation and the development of neoplasms on the ovarian surface.

Since their inception, these theories have received tremendous epidemiological evidence to support their claims. Indeed, there is a clear link between increasing the number of ovulatory events in a lifetime and the predisposition to developing OvCa. As an example, Purdie *et al* showed that there was an average 6% increase in risk in developing OvCa associated with each ovulatory year; furthermore, they show that the use of oral contraceptives decreased this risk by 8% for each year of ovulation suppressed, and that pregnancy resulted in a 12% reduction in risk associated with each year of ovulation suppressed through full-term pregnancies (Purdie et al., 2003). This study along with many others (Hildreth et al., 1981; Mori et al., 1988; Risch et al., 1983; Whittemore et al., 1992; Wu et al., 1988) demonstrate the protective effect of pregnancy and oral contraceptive use against developing OvCa. Similarly, tubal ligation and hysterectomies has been shown to be protective against developing OvCa (Hankinson et al., 1993), but contradictory evidence showing that tubal ligation as well as infertility increase OvCa risk put these theories into question (Tworoger et al., 2007). Studies have suggested that the use of fertility drugs may increase OvCa risk, but the evidence is contradictory and no clear consensus has been achieved (Ness et al., 2002; Rossing et al., 1994).

Although most OvCas develop sporadically, there exists a hereditary predisposition to developing OvCa, where a strong family history of either breast or ovarian cancer (breast-ovarian cancer family syndrome) exists. Breast-ovarian cancer syndrome accounts for 10 – 15% of all OvCas, and is the most important risk factor in developing OvCa (Cannistra, 2004; Pal et al., 2005; Risch et al., 2001; Rubin et al., 1998), although an identifiable genetic predisposition is present in only 5% of affected women in this group (Cannistra, 2004). It is though that this hereditary predisposition is in part due to germ-line mutations in the breast cancer 1 (*BRCA1*) or breast cancer 2 (*BRCA2*) genes, where the lifetime risk of developing OvCa in women with a *BRCA1* mutation is 40%, whereas a *BRCA2* mutation results in a 10-20% lifetime risk. Another hereditary disorder associated with increased risk of OvCa is called the Lynch syndrome II, which stems from inherited mutations in DNA mismatch repair genes. Patients suffering from this syndrome also have an increased risk of developing colon, endometrial and gastric cancers (Lindor et al., 2006).

1.1.4 OvCa Screening

Although detection of early-stage OvCa has been associated with a better prognosis and longer disease-free survival, there is currently no screening program available to detect OvCa at an early stage (Williams et al., 2007). Many studies have been undertaken to evaluate the predictive value in using cancer antigen 125 (CA125) serum levels or transvaginal ultrasound as an independent screening test (Urban, 1999). Transvaginal ultrasound, even though highly sensitive at detecting pelvic masses, is not very specific at detecting tumorous masses, and as such cannot be used as an independent testing modality for detection of OvCa. This low predictive value is primarily due to the

commonality of benign pelvic lesions present in women (Aletti et al., 2007). The only FDA-approved blood test for the detection of OvCa is the CA125 test, which detects serum levels of CA125, a cell surface glycoprotein belonging to the mucin family and is typically elevated in advanced stages of OvCa. This test is not very useful in the detection of OvCa at early stages, because it has been shown that only 50% of women with Stage I OvCa have CA125 levels that are above the elevated threshold level of 35 U/mL, while CA125 levels are above threshold in 82% of women with advanced stage disease (Aletti et al., 2007; Jacobs et al., 1996; Williams et al., 2007). Additionally, many health conditions other than OvCa can lead to elevated CA125 levels, such as uterine fibroids, endometriosis, menses, pregnancy and pelvic inflammatory disease, as well as other cancers such as pancreatic, breast, lung, gastric and colon cancers (An et al., 2006; Cannistra, 2004). However, once the patient has been diagnosed with OvCa, the monitoring of serum CA125 levels is very effective at predicting patient outcomes and response to treatments, and is the primary indication for the use of this serum test (Aletti et al., 2007; An et al., 2006; Cannistra, 2004; Jacobs et al., 1996). Therefore the final diagnosis for OvCa still involves invasive abdominal surgery. Currently two large randomized studies are assessing the predictive value in a combination screening strategy consisting of annual pelvic examination, transvaginal ultrasound and CA125 serum levels for detecting OvCa in asymptomatic patients (Aletti et al., 2007); these studies will hopefully answer the largely debated question of whether ultrasound with or without CA125 screening can increase the percentage of early detections.

With the recent development of technologies such as genomic, metabonomic and proteomic platforms that can more easily screen the differential gene or protein

expression of many molecules simultaneously between normal and diseased states, the race to discover the perfect OvCa biomarker is moving at an incredible pace, yielding some promising candidates. A recent gene array study undertaken by Lu *et al* studied global gene expression patterns between 42 normal tissue samples and 42 OvCa patient samples at different stages, grades and histotypes (Lu et al., 2004). Expression levels of claudin-3 (CLDN3), CA125, mucin 1 (MUC1) and vascular endothelial growth factor (VEGF) were able to distinguish between normal and cancerous cells, demonstrating that the use of a panel of gene biomarkers may be a useful and a more reliable screening strategy as compared to one single gene biomarker. Another study conducted by Swisher and colleagues examined whether levels of circulating tumor DNA could be identified by tumor-specific tumor protein 53 (p53) mutations (Swisher et al., 2005). p53 gene mutations are the most common somatic alteration in OvCa and are found in all stages of OvCa (Swisher et al., 2005). The authors identified that of those patients with somatic p53 mutations (69 out of the 137 OvCa tumors tested), tumor-specific p53 sequences were detected in 30% (21) of plasma or serum samples. The peritoneal fluid of a separate cohort of OvCa patients with known somatic p53 mutations was then assessed, and 98% (28 out of 30) of patients tested positive, including the 3 patients with Stage 1 cancer (Swisher et al., 2005). These results demonstrate the potential to detect the DNA for a tumor suppressor gene in serum to become a biomarker for OvCa.

Metabolites have also been studied for the early detection of OvCa. Of these, lysophosphatidic acid (LPA) and lipid-associated sialic acid (LSA) have been the most studied (Williams et al., 2007). LPA has been shown to be elevated in 90% of Stage I and all of the Stage II-IV OvCas (Xu et al., 1998); these results were later corroborated

by Sutphen *et al* in 2004 (Sutphen et al., 2004). On the other hand, studies for the use of LSA as a potential OvCa metabolite marker have been conflicting due to the inconsistent data obtained assessing its predictive value as a biomarker, and put into question the validity of this metabolite in serving as an effective marker for OvCa (Katopodis et al., 1982; Petru et al., 1990; Schutter et al., 1992; Stratton et al., 1988; Vardi et al., 1989).

The majority of OvCa biomarker research has focused on the development of protein biomarkers, as proteins are essentially the end products of gene transcription and are the effectors of altered states in the body, being healthy or diseased. A large number of proteins have been identified as potential OvCa marker candidates. Among these is Haptoglobin, where a number of studies have demonstrated its ability to differentiate between normal and OvCa patient serum samples with high sensitivity and specificity (Ahmed et al., 2004; Ye et al., 2003). The addition of serum CA125 levels further increased its positive predictive value (Ye et al., 2003), demonstrating the potential utility of this protein, either alone or in combination, to detect OvCa. Furthermore, Mor *et al* demonstrated that the combined expression profile of 4 serum protein biomarkers: leptin, prolactin, osteopontin and insulin-like growth factor II (IGFII) were capable of diagnosing OvCa, with a 95% specificity and sensitivity could be achieved; this means that this test had a low rate of false-positives (highly specific) along with a low rate of false-negatives (highly sensitive) (Mor et al., 2005).

Although the research on OvCa biomarkers is yielding exciting results, to date, there is no single gene, metabolite or protein that outperforms CA125 in its ability to detect OvCas (Williams et al., 2007). Additionally, no two independent proteomic studies have identified the same proteins as potential OvCa markers. Further research is

needed to discover new biomarkers, but to also investigate the combination of already identified gene, metabolite and protein biomarker expression profiles in the hopes of improving the accuracy of early OvCa detection.

1.1.5 OvCa Treatments For Primary And Recurring Disease

The standard of treatment for advanced stage OvCa patients is a combination of cytoreduction surgery and chemotherapy. Early stage disease always involves cytoreductive surgery, but may involve a number of different surgical procedures to remove tumorous or suspicious masses depending on the spread and aggressiveness of the cancer. These procedures can involve total abdominal hysterectomy (removal of the uterus), infracolic omentectomy (excision of the omentum beneath the transverse colon), bilateral salpingooophorectomy (removal of the fallopian tubes and ovaries), and lymph-adenectomy (lymph node dissection) (Williams et al., 2007). Several studies suggest that adjuvant chemotherapy for early stage (Stages Ia to IIc), low-risk patients is not needed (Young et al., 1990), however adjuvant chemotherapy immediately after reduction surgery is now recommended for early stage high-risk patients based on two large randomized trials conducted by the International Collaborative Ovarian Neoplasm (ICON-1 trial) (Colombo et al., 2003) and the European Organization for Research and Treatment of Cancer (ACTION trial) (Trimbos et al., 2003b). Results showed that there was a statistically superior overall 5 year survival and progression-free survival in the adjuvant chemotherapy group as compared to the non-adjuvant chemotherapy group (Trimbos et al., 2003a).

As previously mentioned, first-line therapy for advanced stage OvCa patients (IIIa – IV) always involves aggressive cytoreductive surgery in combination with adjuvant

chemotherapy. This chemotherapy regime usually consists of a combination of drugs such as platinum-containing drugs (i.e. cisplatin, carboplatin or oxaliplatin) along with taxol (palcitaxel) (Aletti et al., 2007; Bukowski et al., 2007; Williams et al., 2007). Cisplatin and carboplatin are crosslinking agents, which form inter- and intra-strand cross-links with DNA or RNA, effectively disrupting DNA replication and transcription. The formation of DNA adducts will produce DNA damage beyond repair, inducing cellular apoptosis (Boulikas and Vougiouka, 2003; Piccart et al., 2001). Oxaliplatin is a third generation platinum derivative that was approved by the FDA for the treatment of colorectal cancer in 2002. As with cisplatin and carboplatin, oxaliplatin cross-links DNA and RNA and adducts are formed, but the structure of the adducts differ and will be recognized by different DNA repair proteins (Fu et al., 2006). Because of its different mechanism of action, oxaliplatin is capable of overcoming the cisplatin resistance in ovarian cancer cells, in addition to producing a synergistic cytotoxic effect in combination with other chemotherapeutic agents. Thus oxaliplatin is an attractive agent for use in combination therapy and in cisplatin-resistant patients (Fu et al., 2006). Paclitaxel is a microtubule stabilizer, and will cause the hyperstabilization of the cell's cytoskeleton during mitosis, leading to an inhibition of cell division (Williams et al., 2007). These chemotherapeutics are usually given intravenously (IV); however, there has been some recent discussion and study about the potential benefit of administering chemotherapeutic drugs intraperitoneally (IP) in women with optimally debulked disease. Large phase III trials have shown statistically significant improvements in both progression-free and disease-free survival. Nevertheless, significant complications due to IP-catheter placement have translated to poor patient compliance where most patients

could not finish their course of chemotherapy; these complications question the practical use of this delivery method and has limited its adoption in the clinic (Rao et al., 2007).

Although women respond well to IV platinum and taxol initially, 50-75% of patients will have a recurrence of their OvCa within 5 years. For reasons that are not yet understood, a large proportion of those recurrences are now resistant to conventional chemotherapeutics. Unfortunately, most recurring OvCas are incurable, and the mean survival rate after recurrence is 2 years (Ozols, 2005). Thus the primary goal of therapy for recurrent disease is symptom management. Patients with platinum-resistant disease have a number of agents that provide effective symptom management, including topocenan, taxanes, doxorubicin and etoposide. Additionally, hormonal therapies have been used for treating recurrent OvCa patients with some success; tamoxifen, ethinyl estradiol and medroxyprogesterone have shown modest response rates between 6 and 17% (Aletti et al., 2007). However, all recurrent OvCa therapies show response rates below 20%, and therefore much research has been undertaken to study new therapeutic strategies. Some promising therapies that are currently being investigated in clinical trials include Herceptin (Trastuzumab), a monoclonal antibody to block v-erb-2 erythroblastic leukemia viral oncogene homolog 2/neuro/glioblastoma derived oncogene homolog (erbB2/neu/HER2) receptor kinase activation involved in stimulating epithelial cell proliferation, Lapatinib, a potent dual inhibitor of epidermal growth factor receptor (EGFR) and erbB2/neu/HER2 receptor activation, as well as Avastin (Bevacuzimab), another monoclonal antibody used to block the actions of VEGF, a potent stimulator of angiogenesis in OvCa cells (Aletti et al., 2007; Williams et al., 2007). Recurring OvCa accounts for the majority of deaths due to OvCa, thus the discovery of chemotherapeutic

agents to prevent, or effectively treat recurring OvCa is of crucial importance in order to improve patient prognosis.

1.1.6 OvCa Carcinogenesis And Tumor Progression

Ovarian surface epithelial lesions have been widely accepted as precursors to the development of OvCa. Two main types of lesions have been described as precursors of OvCa: lesions that appear *in situ* in the OSE or in inclusion cysts that potentially give rise to high-grade tumors, and pre-existing benign ovarian tumors and endometriosis that may give rise to low-grade OvCas (Bell, 2005; Shih Ie and Kurman, 2004). The first theory of how OvCa develops was forwarded by Fathalla, who postulated that the repeated and cyclic process of ovulation caused repeated damage to the OSE, predisposing these cells to the accumulation of genetic changes and the development of neoplastic changes leading to the formation of OvCa (Fathalla, 1971). Support for this hypothesis has been shown in the experimental setting, where rapid cell division in the OSE during the 24 h period after ovulation can encourage chromosomal aberrations (Williams et al., 2007), and neoplastic transformation of the OSE has been shown to occur in cultures of rat and human OSE (Auersperg et al., 2001). Another view of OvCa carcinogenesis was extended by Stadel, who put forth the gonadotropin hypothesis, where the ovarian surface forms inclusion cysts derived from crypts or invaginations of the OSE. The exposure of the enclosed OSE cells to elevated levels of pituitary gonadotropins such as follicle-stimulating hormone (FSH) and luteinizing hormone (LH) will promote the increased generation of estrogen, leading to stimulation of rapid cell division and malignant transformation of the OSE contained within the cyst (Stadel, 1975). Support for this hypothesis has been shown experimentally, where gonadotropin

receptors are expressed in both normal and OvCa cells, and that normal OSE cell cultures can respond to gonadotropins (Auersperg et al., 2001; Wong and Leung, 2007). However, there are a number of growth factors and hormones that are produced by the OSE (discussed later), and many of these signalling molecules maintain autocrine pathways that could potentially have a tumorigenic impact on the OSE that is confined within a cyst (Auersperg et al., 2001). Additionally, the stroma surrounding the inclusion cyst is also a potential source of growth factors that could act in a paracrine fashion to influence OSE cells contained within the cyst (Ahmed et al., 2007).

There is some debate on whether inclusion cysts solely arise from ovulatory events; the fact that large numbers of cysts were found in women with decreased ovulation and women with multiple pregnancies (Scully, 1995) suggests that cyst formation can be a result of ovulation, but is not dependent on the number of ovulatory events and thus can form through other mechanisms that are currently unknown. However, there is mounting evidence to suggest that ovarian cysts are sites of neoplastic transformation. Salazar and others studied ovaries removed by prophylactic oophorectomy in women at high risk of developing familial OvCa, and identified the presence of inclusion cysts and small neoplastic lesions within these ovaries (Salazar et al., 1996). Furthermore, a number of molecular changes have been seen in the epithelial cells lining inclusion cysts, such as p53 and CA125 overexpression (Nnene et al., 2004; Scully, 1995), suggesting that genetic abnormalities can occur in inclusion cysts that may predispose the cells to malignant transformation.

Pre-existing benign ovarian tumors and endometriosis have also been accepted as potential precursors of OvCa. It is thought that benign tumors and endometriosis may

give rise to low grade serous and endometrioid OvCa, as these tumors have a low proliferative index and tend to remain undetected for up to 20 years (Shih Ie and Kurman, 2004). It has also been shown that 15-50% of clear cell and endometrioid carcinomas are associated with endometriosis in the ovary or pelvis (Smith Sehdev et al., 2003), leading researchers to postulate that endometriosis may be a precursor to these tumors. Furthermore, a recent study using transvaginal ultrasound determined that approximately 50% of OvCas develop from pre-existing benign tumors, and that the remaining 50% develop in ovaries without apparent abnormality (Horiuchi et al., 2003b). Analysis of genetic alterations in these tumors revealed that in opposition to high-grade serous tumors which have frequent p53 mutations, low-grade clear cell and endometrioid tumors have frequent v-raf-murine sarcoma viral oncogene homolog B1 (BRAF), v-ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS), β-catenin and phosphatases and tensin homology (PTEN) mutations (Shih Ie and Kurman, 2004). These studies have led to the development of a dualistic model of OvCa tumorigenesis based on epidemiological and molecular genetic analyses: The Type I tumors, comprised of low-grade serous, mucinous, endometrioid and clear cell carcinomas, develop in a stepwise manner through well-recognized precursors such as benign lesions and adenofibromas. These tumors possess known genetic alterations (*BRAF*, *KRAS*, *β-catenin* and *PTEN*) and are slow-progressing, with a better prognosis. Type II tumors represent high-grade serous and undifferentiated tumors that are rarely associated with morphologic precursor lesions, and develop from the OSE or inclusion cysts. They have very frequent p53 mutations, evolve rapidly, metastasize early and are highly aggressive, with poor prognosis. It is also

thought that 25% of serous OvCas are Type I tumors, and 75% of serous OvCas are of Type II (Bell, 2005; Shih Ie and Kurman, 2004).

There is some evidence to suggest that the epithelial lining of the fallopian tube is the site of origin for some OvCas (Crum et al., 2007; Lee et al., 2007). A study found that high-risk women who had prophylactic salpingo-oophorectomy due to BRCA1 and BRCA2 germline mutations showed carcinoma *in situ* in the fallopian tubes, while no cellular changes were seen in the ovaries, suggesting that BRCA carriers may show OvCas originating from the fallopian tube epithelium; however, further patient testing will need to be conducted in order to confirm this link (Piek et al., 2003a; Piek et al., 2003b).

OvCa first spreads by direct extension into adjacent organs of the reproductive systems, especially the fallopian tubes, uterus, and occasionally the bladder, pelvic sidewall and rectum (Tan et al., 2006). After direct extension, the OvCa cells can disseminate through three different routes: transcoelomic, haematogeneous or lymphatic. Transcoelomic metastasis (into the peritoneal cavity) is the most common route of dissemination, which occurs in 70% of patients whose OvCa has metastasized (Tan et al., 2006). This route of metastasis also accounts for the morbidity associated with this type of cancer, as it has the capacity of affecting a number of organs within the peritoneal cavity. The most widely accepted explanation for the mechanism of peritoneal metastasis is that tumor cells become detached from the primary tumor after extension, and are transported by passive mechanisms throughout the peritoneal cavity before re-seeding to other organs. However emerging evidence points toward an alternative view where OvCa cells show many instances of adaptive behaviour in order to effectively survive

within the peritoneal fluid and re-attach to foreign surfaces with the end result of metastatic spread. Tan *et al* suggest a 6-step process of OvCa metastatic spread; Step 1 involves anchorage-independent growth through the upregulation of the vesicular trafficking protein Ras oncogene family 25 (*RAB25*), involved in preventing apoptosis and anoikis. OvCa cells have also been shown to undergo an epithelial-mesenchymal transition (EMT) *in vitro*, with an upregulation of the EMT marker Snail, and a down-regulation of the cell-cell junction protein E-cadherin, which could confer cellular motility and invasive capabilities to the cancer cells (Rosano et al., 2005; Theriault et al., 2007) *in vivo*. OvCa cells undoubtedly need to alter their adhesive capabilities in order to detach from the primary tumor, and evidence shows integrin receptor upregulation in malignant but not normal ovarian tissue (Ahmed et al., 2002). Furthermore, upregulation of urokinase-like plasminogen activator receptor (uPAR), with its subsequent engagement of integrin receptors and cellular detachment, has also been implicated in OvCa cell progression (Czekay and Loskutoff, 2004). Therefore OvCa cells have shown to display changes in behaviours that are crucial for the initial steps of metastasis.

The second step involves dissemination throughout the peritoneal cavity. The long-held view of peritoneal metastasis is that the flow of peritoneal fluid would direct the OvCa cells to distant organs where they could form tumor nodules. The fluid flow of the peritoneal cavity is directed by gravity, and follows a clockwise flow towards the anterior portion of the cavity due to the negative pressure caused by respiration. One would thus expect that the distribution of the OvCa cells would follow this directional flow of fluid, and would see a larger number of cancerous implants in the lower portions of the pelvis (such as the omentum) and on the surface of the diaphragm. Indeed this is

the case; in a prospective study which observed the distribution of peritoneal metastases in patients with various tumors including OvCa, the omentum, lower colon and diaphragm were most commonly affected (Carmignani et al., 2003).

The third step involves evasion of the immune system. OvCa cells would need to acquire mechanisms for them to circumvent immunological surveillance in the peritoneal cavity in order to properly disseminate. This function could be performed by the formation of ascitic fluid, which is the host's immunologic reaction to the presence of malignant cells within the peritoneal cavity. Many studies have pointed to the blockage of proper lymphatic drainage by tumor cells as a mechanism of ascites formation, but other mechanisms such as edema caused by increased capillary permeability within the peritoneal wall vasculature are also plausible (Aslam and Marino, 2001). This ascitic fluid has been shown to contain many immune modulators such as tumor necrosis factor superfamily member 6 (FAS ligand), complement inhibitors and alternative pathway inhibitors factor H and factor H-like protein 1, as well as the recruitment of regulatory T cells to suppress tumor-specific T-cell immunity (Tan et al., 2006). Cytokines and growth factors that modulate immune responses such as interleukin 1 (IL-1), 6 and 8, macrophage colony stimulating factor (M-CSF), epidermal growth factor (EGF), hepatocyte growth factor (HGF) and TGFß have also been found in ascitic fluid and expressed on OvCa cells; additionally our laboratory has shown that TGFß is present in the ascites obtained from OvCa patients (Dunfield and Nachtigal, 2003; Tan et al., 2006; Wong and Leung, 2007), indicating that the presence of ascites provides an immunologically privileged microenvironment for the metastatic progression of OvCa cells.

Further support for the adaptive metastatic process in OvCa is shown through the last three steps of tumor progression, where OvCa cells form small aggregates called spheroids, attach to peritoneal surfaces and invade through the mesothelial cell layers to form tumor nodules. OvCa cell spheroids have been isolated from ascitic fluid and primary OvCa cells isolated from ascites can form spheroids *in vitro* (Shepherd et al., 2006; Tan et al., 2006). This may be another mechanism through which the OvCa tumor cells can travel through the peritoneal fluid and evade immune detection, as it has been shown that *in vitro* spheroids show increased complement resistance (Tan et al., 2006). Chemokine receptors such as the chemokine (C-X-C) motif receptor 4 (CXCR4) expressed on OvCa cells can bind to its ligand, chemokine (C-X-C) motif ligand 12 (CXCL12) present in ascitic fluid and mediate increased migration through the peritoneal cavity and attachment to peritoneal surfaces (Scotton et al., 2001). It is thought by some researchers that in order for OvCa spheroids to propagate onto peritoneal surfaces, they undergo a mesenchymal to epithelial transition (MET) response where they re-adopt certain epithelial characteristics such as upregulation of the cell-cell adhesion receptor E-cadherin in order to establish metastases (Ahmed et al., 2007). In fact, E-cadherin levels have been shown to fluctuate during OvCa progression and metastasis, where OSE cells express low levels of E-cadherin, but gain E-cadherin expression during OvCa progression, only to loose it again at late stages of OvCa metastasis (Auersperg et al., 2001; Rosano et al., 2005; Theriault et al., 2007; Zeineldin et al., 2006). After attachment of spheroids to peritoneal surfaces, which involves integrin receptor engagement as well as the upregulation of the tumor-associated glycoprotein CA125 and E-cadherin, they can undergo another EMT response and disaggregate, proliferate and invade the mesothelial

layers lining the peritoneal cavity through loss of E-cadherin, integrin receptor activation, and expression of matrix metalloproteases (MMP) and uPA/uPAR (Burleson et al., 2004a; Burleson et al., 2004b; Tan et al., 2006). It is therefore apparent that OvCa cells actively participate in the metastatic process, and as the cell types from which they arise (OSE), maintain inherent aspects of cellular plasticity that allow them to adapt to a number of extracellular environments in order to successfully metastasize within the peritoneal cavity.

1.1.7 Experimental Models Of OvCa

A number of *in vitro* and *in vivo* models have been developed to study the mechanisms of OvCa development, progression and metastasis. *In vitro* analyses comprise the study of OvCa cell lines, or primary OvCa cells obtained from tumor tissues or ascites fluid collected from OvCa patients. There are advantages and limitations to the use of these systems; OvCa cell lines, although having the advantage of almost unlimited replicative capacity, contain many genetic alterations that differ from the primary tumor and may affect the relevance of results to *in situ* cellular biology. This is especially true when studying the effects of various agents on cellular proliferation since a common genetic difference between primary tumor cells and established OvCa cell lines is the deletion of the cyclin dependent kinase $p15^{Ink4B}$ by the cell lines (Dunfield et al., 2002a; Ichikawa et al., 1996; Watson et al., 1999). Primary OvCa cells obtained from patients on the other hand, are likely more reflective of the disease processes *in vivo* and the patient population than OvCa cell lines, but also may contain alterations due to their forced adaptation to cell culture conditions, as well as present variability in responses due to the heterogeneous nature of human OvCas. The limited detection of early-stage

OvCas makes it so that most primary OvCa cells are obtained from patient tumors or ascitic fluid at advanced stages of disease and are not suitable to study early events in OvCa pathogenesis. Many researchers have circumvented the restrictions of two-dimensional monolayer cultures by growing cells in suspension, spheroids, or in gel matrices that support three-dimensional growth; these methods have advantages as they more closely replicate the *in vivo* nature and behaviour of metastatic OvCa cells that reside within the peritoneal fluid. The ability to isolate and culture normal OSE cells from human and animal ovaries has been crucial in understanding the early events of OvCa progression, and to perform comparative analyses between normal and cancerous cells in the hopes of identifying novel biomarkers and/or signalling pathways involved in OvCa tumorigenesis (Auersperg et al., 1984; Burleson et al., 2006; Dunfield et al., 2002b; Nakamura et al., 1994; Ohtake et al., 1999; Shepherd et al., 2006; Zietarska et al., 2007).

The longest-standing and most popular method of studying tumor formation *in vivo* is human OvCa cell xenografts, where human OvCa cells are inoculated into immunodeficient rodents either through subcutaneous (SC), IP or orthotopic administrations. The obvious pitfall to these procedures is that the animal models are immunocompromised, and so the immune component to cancer progression cannot be studied. Rodents have a unique anatomical organization in that they possess a bursal membrane, which is a sac that surrounds the ovary and the oviduct, and provides an opportune environment for the intrabursal injection of cells or agents that will be confined within this space which is in close proximity to the OSE (Garson et al., 2005). This procedure may not be optimal for studying metastatic spread, as the cells and/or

agents are confined to the ovary, but may be useful in studying early events in OvCa progression.

Many genetic models of OvCa have been developed in recent years through transgenic mouse models. The first successful transgenic model for OvCa was reported by Connolly *et al*, where they expressed the early region of simian virus 40 (SV40) T Antigen under the control of the müllerian inhibiting substance receptor type II promoter (MISIIR; tgMISIIRTAg), a receptor that had been previously shown to be upregulated in OvCa cell lines and cells isolated from patient ascites (Connolly et al., 2003; Masiakos et al., 1999). Approximately 50% of the transgenic mice developed ovarian tumors within 6-13 weeks of age, and were associated with the frequent appearance of ascites, but upon histological observations were identified as poorly differentiated carcinomas. Furthermore, the MISIIR promoter also shows strong expression in granulosa cells as well as OSE, and so may not be an appropriate model to study cancers solely arising from the OSE.

The difficulty in finding suitable promoters for the OSE-specific expression of various genes in transgenic models has been partially circumvented by the exploitation of the Cre-loxP system. This system involves the engineering of the gene of interest with flanking loxP sites (a floxed gene), which are targets for the Cre recombinase. If the loxP sites are engineered to point in the same direction on either side of the flanked sequence, then Cre recombinase will bind to these loxP sites and will irreversibly excise the DNA of interest and recombine the remaining sequence. This effectively produces a conditional knockout animal, where the gene will be "knocked out" only when and where the Cre recombinase is present. In the same manner, the genome can be modified to insert

transcriptional stop sequences flanked by loxP sites ("lox-stop") between the promoter and the transgene; this will result in expression of the gene only when Cre recombinase is present to excise the lox-stop sequence and recombine the native gene sequence. The expression of Cre can be OSE-specific through intrabursal injection of a viral vector expressing Cre recombinase. This strategy has been employed in at least three animal models to knock out the tumor suppressor genes *p53* and tumor protein retinoblastoma (*pRb*) (Flesken-Nikitin et al., 2003), to knock out *PTEN* and activate *KRAS* simultaneously (Dinulescu et al., 2005), and to inactivate the *BRCA1* gene (Clark-Knowles et al., 2007). Although all of these models have proved invaluable in identifying changes that are critical for tumorigenesis of the OSE, none of them can exactly recapitulate all of the various forms of human OvCa that exist. Thus further refinement of these models and the generation of new genetic models will undoubtedly further our understanding of OvCa tumorigenesis and progression.

1.2 Biology Of The OSE

As mentioned previously, most OvCas are thought to arise from the OSE (Auersperg et al., 2001). The OSE, also referred to as the ovarian mesothelium (Nicosia, 1991), is composed of a single layer of squamous-to-cuboidal epithelial cells that covers the entire surface of the ovary (Auersperg et al., 2001) (Figure 1-1). During embryogenesis, the OSE is derived from the coelomic epithelium. The OSE rests on a basement membrane composed of various ECM proteins such as laminin and several collagens. Albeit existing as a cell monolayer that is inconspicuous in its nature, the OSE participates in transport of growth factors, cytokines and hormones to and from the

peritoneal cavity, and is a crucial player in the process of ovulation (Auersperg et al., 2001).

1.2.1 The Role Of OSE During Ovulation

During each reproductive cycle, the OSE lining the mature follicle undergoes apoptosis and is lost during follicular rupture and release of the egg (Murdoch, 1995). The OSE has also been shown to produce proteolytic enzymes to degrade and weaken the ovarian surface tissue above the maturing follicle to facilitate its rupture (Kruk et al., 1994). A repair process is initiated immediately after ovulation, where the OSE adjacent to the site of ovulation participates in a wound-healing response. This response involves proteolytic activity to degrade the wounded area, and a remodeling activity to re-establish the integrity of the OSE, the basement membrane, as well as some of the underlying stroma. These remodeling activities include growth factor and cytokine production, cellular proliferation, cellular migration, production of ECM and adhesive proteins by the OSE to re-adhere to the newly formed basement membrane. The OSE cells must therefore change their phenotype from a stationary epithelial monolayer to an active remodeling entity during ovulatory repair. It is thought that the OSE is a less differentiated epithelial cell type as compared to the epithelia seen in other coelomic derivatives, where these cells maintain both epithelial (expression of cytokeratins and tight junctions) and mesenchymal characteristics (loss of E-cadherin and expression of vimentin) (Czernobilsky et al., 1985) and therefore have the capacity to respond to a variety of extracellular stimuli and modify their behaviour accordingly. It is therefore thought that this undifferentiated phenotype allows for the OSE to undergo an EMT-MET response during post-ovulatory repair, where OSE cells will temporarily adopt

fibroblastic characteristics such as spindle-shaped morphology, increased contractile activity, migration, secretion of ECM proteins and increased adhesive capabilities through integrin receptor expression (Auersperg et al., 2001). Support for this is based on *in vitro* evidence showing that in cell culture, a number of different growth factors including EGF and TGFß can induce epithelial cells (including OSE) to undergo an EMT response (Auersperg et al., 2001; Salamanca et al., 2004). Additionally, growth in 3-dimentional matrices will also stimulate an EMT in epithelial cells (Berchuck et al., 1992; Toda et al., 1997). Following post-ovulatory repair it is thought that the OSE cells involved in the repair are capable of reverting to their initial epithelial phenotype through a MET (Ahmed et al., 2007). These adoptive processes highlight the plastic and highly adaptable nature of the OSE which allow for cyclical epithelial-mesenchymal interconversions that are tightly controlled (Ahmed et al., 2007). Conversely, the ability of the OSE to exhibit various cell morphologies ranging from squamous to cuboidal (Figure 1-1) suggests that OSE cells may show limits to its ability to revert back to a simple epithelial morphology following postovulatory repair. A study of the cell morphology exhibited by the OSE post-ovulation showed two groups of OSE cells; one group of cells that had not participated in ovulation exhibited cuboidal cell morphologies, while the cells found adjacent to the ovulation site exhibited squamous cell morphologies (Gillett et al., 1991). These findings suggest that the cyclic process of wound repair following ovulation may in some way alter the biology of the OSE over time, as well as its responses to the environment. It is thought that either the accumulation of genetic aberrations and/or imbalances in the regulation of these processes in OSE cells may allow them to acquire differential responses and behaviours to pathologic extracellular

influences with the end result of neoplastic progression (Auersperg et al., 2001; Wong and Leung, 2007) (discussed below).

1.3 Regulation Of OSE And OvCa By Hormones, Cytokines And Growth Factors

A variety of molecules are expressed in both OSE and OvCa cells, and many of these factors maintain autocrine and/or paracrine signalling loops, suggesting that they may not only control normal OSE biology, but may also have a role to play in the development or maintenance of OvCa.

1.3.1 Peptide Hormones

Normal OSE and OvCa cell lines express both forms of Gonadotropin-releasing hormone (GnRH-I and -II) as well as the GnRH receptor (GnRHR), and both ligands show a growth inhibitory effect on normal OSE and OvCa cell lines by acting through the GnRHR (Kang et al., 2000). The expression of GnRH-I and –II and the GnRHR is regulated by sex steroids (estrogen, progesterone, testosterone) and peptide hormones (activin, inhibin, gonadotropin) (Cheng and Leung, 2005). Gonadotropin receptors such as for FSH and LH are expressed in a variety of normal and immortalized OSE cells, as well as OvCa cell lines. Additionally, normal OSE and OvCa cell lines can respond to both FSH and LH stimulation, but experimental results have been conflicting. While some reports show that FSH and LH stimulate OSE proliferation (Choi et al., 2002), other reports show either no effect or growth inhibition (Ivarsson et al., 2001). The theory behind this differential response may be due to the concentration of the ligands present; evidence shows that a surge in FSH levels causes oocyte cellular apoptosis, while lower levels induces cellular proliferation (Thomas et al., 2005). Cyclic AMP (cAMP)/ protein kinase A (PKA), mitogen-activated protein kinase (MAPK) and IL-6/

signal transducer and activator of transcription 3 (STAT3) signalling pathways have all been involved in transducing the growth-regulatory effects of gonadotropins (Choi et al., 2002; Syed et al., 2002). Recent evidence has also shown that gonadotropins have indirect effects on OSE proliferation via cross-talk with other growth factors. FSH has been shown to stimulate levels of growth-promoting factors such as keratinocyte growth factor (KGF), HGF, and Kit ligand (kitL) mRNA. Additionally, FSH and LH increased EGF receptor mRNA and protein in preneoplastic OSE, indicating a putative mechanism of action for the growth-promoting effects of gonadotropins (Ji et al., 2004). In addition to stimulating proliferation, gonadotropins have also been reported to enhance tumor angiogenesis and adhesion by regulating the expression of vascular endothelial growth factor (VEGF), integrin αV, and CD44 in OvCa cells (Wong and Leung, 2007), suggesting a novel role for gonadotropin signalling in OvCa cell metastasis.

As mentioned earlier, sex steroids such as estrogen, progesterone and androgens are involved in OSE and OvCa cell biology. Receptors for these hormones have been found at the mRNA and/or protein level in OSE and OvCa cells. Estrogenic effects are mediated through estrogen receptor alpha (ERα) and ERß, and there is evidence pointing to the differential expression of these receptors in normal versus neoplastic OSE (Brandenberger et al., 1998) which may explain some of the enhanced growth stimulatory effects of estrogens on OvCa cells. Estrogen has also been shown to play a role in OvCa cell-ECM interactions by upregulating the expression of cathepsin D, fibulin-1 and fibronectin (Wong and Leung, 2007), indicating that estrogenic stimuli may play a role in OvCa-mediated adhesion during metastasis. Progesterone, on the other hand, has been shown to be growth-inhibitory in normal OSE and OvCa cells through the induction of

apoptosis (Bu et al., 1997; Syed and Ho, 2003). Progesterone receptors A (PR-A) and PR-B are expressed in normal OSE and OvCa cells, but a reduction in PR-A was found in OvCa cells are compared to normal OSE cells, while the expression of PR-B remained unchanged (Karlan et al., 1995; Lau et al., 1999). How this differential expression may contribute to OvCa tumorigenesis is unknown. Progesterone has also been shown to inhibit estrogen-mediated OSE cell proliferation, as well as induce OSE cell apoptosis through differential regulation of TGFß isoform expression (Murdoch and Van Kirk, 2002; Rodriguez et al., 2002). High circulating levels of androgens have been associated with an increased risk of developing OvCa (Helzlsouer et al., 1995), and enhanced cell proliferation in response to dihydrotestosterone has been reported for normal and malignant OSE (Edmondson et al., 2002). The mechanism behind how androgens may increase OvCa risk are unknown; one possibility could be through an indirect mechanism where androgens decrease TGFß receptor expression, and as a consequence suppress the growth-inhibitory effects of TGFß (Evangelou et al., 2000).

1.3.2 Cytokines

Cytokines play an important role during the ovulatory process, and it has been shown that cytokines such as IL-1, IL-6, IL-18, M-CSF, and tumor necrosis factor alpha (TNFα) are expressed and secreted as bioactive forms in cultures of normal OSE and are present in the ascitic fluid of OvCa patients (Auersperg et al., 2001; Punnonen et al., 1998). IL-1α stimulates the growth of OSE cells, can regulate the production of ovarian steroids and also stimulates OvCa cell proliferation (Rae et al., 2004). TNFα and IL1-ß can regulate the release of uPA and MMP-9, which is crucial in the process of OSE basement membrane degradation and remodeling during ovulation (Yang et al., 2004).

These results suggest that activation of these cytokines or their loss of function may play an important role in the neoplastic progression of OvCa (Bandera et al., 2003).

1.3.3 Growth Factors

EGF is expressed by OSE cells, and plays a significant role in OSE biology. During follicle rupture, EGF that is released from follicular fluid, blood and stromal cells, comes into contact with adjacent OSE cells and induces cellular proliferation (Maruo et al., 1993). EGF also induces potent EMT responses in OSE and OvCa cells in culture (Ahmed et al., 2006; Dunfield and Nachtigal, 2003), characterized by morphologic alterations from an cobblestone epithelial morphology to a spindle-shaped fibroblast-like morphology with the formation of F-actin stress fibres, and enhanced motility and secretion of MMP-2 and MMP-9 (Ahmed et al., 2006). These EGF-induced processes are most likely involved in inducing an EMT response in OSE cells during post-ovulatory repair. The EGF receptor, which is a member of the erbB2/neu/HER2 receptor tyrosine kinase family, is expressed in 33-75% of OvCas, and is frequently amplified and/or overexpressed as compared to normal OSE (Berns et al., 1992). Furthermore, EGF has been shown to reduce the growth-inhibitory effect of TGFß in primary human OvCa cells (Dunfield and Nachtigal, 2003), and modulate the growth-stimulatory effect of gonadotropins (Choi et al., 2002). TGFα, a structural analogue of EGF, binds to the same EGF receptor and also stimulates the proliferation of OvCa cells (Auersperg et al., 2001). The heregulin/*neu* differentiation factor binds to receptors within the same erbB2/neu/HER2 receptor tyrosine kinase family as the EGF receptor. Although normal OSE cells express EGF receptors, they do not express HER-2/*neu* in very large amounts; however this receptor is overexpressed in 25-30% of OvCas, and its expression is

associated with a poor prognosis (Auersperg et al., 2001). Therefore elevated expression of either EGF family ligands and/or receptors in OvCa cells may stimulate autocrine or paracrine loops that play an important role in tumor formation and progression. Two drugs are currently under clinical testing for the treatment of advanced-stage OvCa: Herceptin, which is a monoclonal antibody that blocks HER-2/*neu* receptor activation, and Lapatinib, which is a potent tyrosine kinase small molecule inhibitor of EGF receptor and HER-2/*neu* receptor activation. Thus blocking the effects of elevated EGF family ligands or receptors through the use of inhibitors may show promise in slowing OvCa progression.

HGF is primarily produced in mesenchymal and stromal cells in the ovary, and acts through a paracrine loop via the Met receptor expressed on OSE cells (Wong and Leung, 2007). This receptor is encoded by the *c-met* protooncogene (Auersperg et al., 2001). Although normal OSE cells do not express HGF and rely on the paracrine action of HGF-Met production by the stroma to maintain normal ovarian physiology, evidence points to HGF forming an autocrine signalling loop in OvCa cells. Consistent with this, OvCa cells have been shown to express the mRNA for HGF along with Met (Wong et al., 2001b). Additionally, high levels of HGF were found in malignant cystic and ascites fluid as compared to benign ovarian cysts (Baykal et al., 2004; Sowter et al., 1999), and an autocrine HGF-Met signalling loop has been found in OSE cells from women at risk of developing OvCa (Wong et al., 2001a; Wong et al., 2001b), suggesting that the production of HGF by OSE cells may be an early sign of neoplastic progression. Interestingly, the paracrine influence of HGF on the OSE depends on the presence of the basement membrane component fibronectin, as OSE cells in culture will only respond to

HGF in a mitogenic fashion when they are plated onto fibronectin, but not on tissue culture plastic, where they undergo apoptosis in response to HGF (Gulati and Peluso, 1997; Hess et al., 1999). These effects on OSE cells appear to also be regulated by estrogen and gonadotropins (Liu et al., 1994; Negami et al., 1995), highlighting the important influence of hormones and ECM in regulating normal ovarian physiology. HGF has been shown to stimulate the proliferation and motility of OvCa cells, possibly through the activation of extracellular signal-regulated kinase (ERK)/MAPK signalling pathway and a rapamycin-sensitive p70 S6 kinase pathway (Wong and Leung, 2007). Blocking the activity of HGF or Met has been shown to decrease the growth, invasion and metastasis of OvCa cells (Corps et al., 1997; Saga et al., 2001; Ueoka et al., 2000; Zhou and Wong, 2006), suggesting that this autocrine pathway may have an important role in OvCa cell dissemination. Other growth factors that stimulate normal OSE cell growth and may play a role in OvCa cell growth include basic fibroblast growth factor (bFGF), KGF, IGF-I and platelet-derived growth factor (PDGF) (Dabrow et al., 1998; Gubbay et al., 2004; Parrott et al., 2000; Pierro et al., 1996; Trolice et al., 1997), but the most studied family of growth factors that have been shown to greatly affect normal OSE and OvCa cell biology are the TGFß superfamily of ligands, which include, but are not restricted to, TGFß, BMPs and Activins. These three classes of molecules, their signalling mechanisms and their effects on the biology of the OSE and disease processes including OvCa will be the subject of further discussion below.

1.4 Bone Morphogenetic Proteins

BMPs have long been known as multifunctional regulators of tissue morphogenesis, and also play important roles in the maintenance of adult tissues (Chen et

al., 2004). Their activity was first identified in the 1960s as autoinducers of bone formation (Urist, 1965). Since then, over 20 ligands of the BMP signalling family have been identified and characterized, and shown to participate in many functions including tissue patterning of the heart, cartilage and neural tissues during embryogenesis, and bone tissue remodeling and maintenance in adults. BMPs have been involved in the regulation of cell proliferation, survival, proliferation and apoptosis (Chen et al., 2004), but emerging roles for BMPs in other adult tissues such as the ovary are also becoming apparent. Being such crucial regulators in developing and adult tissues, BMPs have also been shown to play significant roles in regulating cellular behaviours in pathogenic situations, including cancer.

1.4.1 BMP Signalling Pathways

BMPs form the largest family of ligands within the TGFß superfamily of ligands. A unique feature of TGFß ligands is that they possess seven conserved cysteine residues, 6 of which are involved in folding the molecule into a cysteine knot (Vitt et al., 2001). The seventh cysteine residue is responsible for forming a disulfide bridge and linking two subunits to form a covalently linked, biologically active dimer (Jones et al., 1994). All TGFß superfamily molecules are translated as large precursor molecules composed of a signal peptide, a prodomain, and a mature domain. After the BMP signal peptide has been removed, dimerization of the proproteins proceeds. Proteolytic enzymes then cleave the BMP dimerized proprotein, resulting in the generation and secretion of biologically active molecules (Shimasaki et al., 2004) (Figure 1-2).

Canonical BMP signal transduction is carried out by ligand binding to a heterotetrameric complex of distinct type I and type II serine/threonine kinase receptor

dimers. The kinase domains of the type II receptors are constitutively active, and phosphorylate the type I receptors upon ligand binding at a conserved Gly-Ser (GS) domain, leading to their activation. The activated type I receptors will then phosphorylate and activate receptor-activated Smads (R-Smads), which will partner with a common Smad 4 and translocate to the nucleus to affect target gene expression (Figure 1-3). The type I BMP receptors include (Activin-like kinase) ALK2 (also called ActR-IA), ALK3 (BMPR-IA), and ALK6 (BMPR-IB), and the type II BMP receptor, called BMPRII. BMPRII has a structure analogous to other type II receptors, except for a long C-terminal tail following the serine/threonine kinase domain (Rosenzweig et al., 1995), which can associate with other signalling molecules such as LIM kinase (LIMK) and Tribbles-like protein 3 (Trb3) (Chan et al., 2007; Foletta et al., 2003; Nohe et al., 2004), showing evidence for BMPs effecting Smad-independent signalling. Binding studies have shown that BMPRII appears to exclusively bind to BMP ligands, including BMP2, 4, 6, 7 and 15, while BMPs 2 and 4 preferentially bind to ALK3 and/or ALK6, and that BMP6 and 7 most readily bind to ALK2 and/or ALK6 (Shimasaki et al., 2004). This apparent cross-reactivity between ligands and receptors highlights the complexity of BMP signalling within cells; this complexity is compounded by the fact that BMP ligands can bind to specific type I receptor subtypes in different cell types and it has therefore been difficult to assign a particular BMP ligand to a particular type I receptor (Shimasaki et al., 2004).

The mechanism of how BMP ligands and the type I and type II receptors associate at the cell membrane to form active signalling complexes is still being clarified, but it has been determined that BMP ligands bind more effectively to type I receptors than type II

receptors, and that the binding of ligands to type II receptors is enhanced with the coexpression of type I receptors (Liu et al., 1995); conversely, type I receptors have been shown to bind ligand without the presence of type II receptors (Wrana et al., 1992). These results indicate that unlike the TGFß ligand, BMPs first bind to type I receptors and this association would then recruit the type II receptor into a signalling complex. Another study probing for the expression of type I and type II BMP receptors at the cell's surface revealed the presence of both heteromeric and homomeric complex formation between the type I receptors ALK3 and ALK6 and the BMPRII on the surface of live cells without the presence of ligand (Gilboa et al., 2000). Additionally, both homo- and heterocomplex formation was increased in the presence of BMP2. These findings suggest the possibility that BMP receptors can signal independently of ligand, effectively maintaining a basal level of signalling in the cell. The fact that type I receptor complexes of homooligmers are present on the cell surface and could associate with BMP ligands implies that type I receptor complexes could independently control BMP signals by either signalling through type II receptor-independent effectors, or decreasing the pool of available type I receptors to associate with type II receptors upon ligand presentation. Taken together, these studies point to the theory that BMP ligands can either associate with pre-formed receptor complexes (PFCs) of type I and type II receptors, or with BMP-induced signalling complexes (BISCs) where the ligand first associates with the high-affinity type I receptor, then recruiting the type II receptor. These two modes of signalling will then transduce the activated BMP receptor signals through different effector pathways. Support for this theory stems from a study by Nohe *et al*, where they show that binding of BMP2 to PFCs leads to the activation of the canonical Smad

pathway, whereas binding of BMP2 to BISCs activates a Smad-independent, p38-dependent signalling cascade (Nohe et al., 2002). Hartung and colleagues extended this theory of BMP signalling to determine that two distinct populations of type I and type II BMP receptors exist within the cell membrane; receptors located in cholesterol-enriched detergent-resistant membrane fractions (DRMs), also termed caveola-like membrane fractions, and receptors located in clathrin-coated pits (CCPs) (Hartung et al., 2006). These membrane regions are actively involved in receptor-mediated endocytic pathways, which are crucial to regulating many growth factor signalling cascades. They determined that both types of BMP receptors underwent constitutive endocytosis through CCPs, but that only type II receptors underwent caveola-like internalization. Additionally, they showed that binding of BMP2 to PFCs induced Smad phosphorylation at the plasma membrane, and that continuation of Smad signalling to affect target gene expression required CCP-mediated endocytosis. Conversely, BMP2 associated with high-affinity BMP type I receptors, which recruited type II receptors to form BISCs that underwent Smad-independent signal transduction through caveola-like internalization (Hartung et al., 2006). These results highlight the intricate role of receptor dynamics at the cell membrane in regulating modes of BMP signal transduction (Figure 1-4).

Once the type I receptors become activated, they can transduce their signals intracellularly through the canonical Smad transduction pathway, or through Smad-independent mechanisms. In the Smad-dependent signalling pathway, the activated type I BMP receptors will associate transiently and phosphorylate the receptor-activated Smads (R-Smads) at the last 2 serine residues in their C-terminal SSXS motif, releasing the auto-inhibitory intramolecular interactions between the highly conserved Mad-

homology 1 (MH1) and MH2 domains and allowing for the subsequent formation hetero-oligomeric complexes with the common-partner Smad (co-Smads), Smad 4 (Heldin et al., 1997). The MH1 domain, which is located at the N-terminal portion of R- and co-Smads is involved in the binding of specific DNA sequences, and the MH2 domain located at the C-terminal portion of these proteins is involved in homo- and heteromeric protein complex formation (Nohe et al., 2004). These domains are separated by a linker region of variable amino acid sequence that contains a PY motif (PPXY sequence) which can associate with a number of WW domain-containing proteins, to regulate Smad activity (Miyazono et al., 2005) (Figure 1-5). Smads 1, 5 and 8 are the R-Smads specifically activated through BMP type I receptors, while Smads 2 and 3 are activated by TGFß and Activin type I receptors (Miyazono et al., 2005). The specificity of R-Smad/type I receptor interactions is determined within a region of the kinase domain of the type I receptor called the L45 loop (Shi and Massague, 2003). The R-Smad/Smad 4 complexes will then translocate into the nucleus where they will regulate the transcription of target genes by interacting directly with DNA at BMP response elements (BRE) and/or with various transcription co-activators and/or co-repressors at target gene promoter regions. Among the various target genes that are affected by BMP signalling, the Id proteins (inhibitor of differentiation or inhibitor of DNA binding) are among the most widely studied (Ogata et al., 1993). Ids are basic helix-loop-helix (bHLH) binding proteins that lack a DNA binding domain, and will interact with a number of ubiquitously-expressed non-bHLH and bHLH transcription factors such as E2A gene products, and sequester them from binding to DNA, effectively inhibiting their actions. BMP-stimulated Id expression has been shown to inhibit MyoD/myogenin and NuroD/neurogenin

transcription, resulting in the inhibition of myogenesis and neurogenesis, respectively (Miyazono et al., 2005). Other BMP target genes also include the inhibitory Smads (I-Smads), Smad 6 and 7 which will negatively regulate BMP signalling (Miyazono et al., 2005).

A number of recent reports have highlighted the contribution of Smad-independent signalling to BMP-induced biologic effects. BMP2 can induce signalling complexes at the cell surface through the association with caveola-like structures, activating Smad-independent effectors such as p38 MAPK with the net result of inducing cellular apoptosis (Kimura et al., 2000). Furthermore, the cytoplasmic tail of BMPRII has been shown to directly interact with the cytoskeletal regulator LIMK, and mediate BMP-dependent, Smad-independent changes in the actin cytoskeleton. These LIMK-induced changes through BMPRII association have been shown to play roles in BMP-induced physiologic behaviours such as dendritogenesis (Lee-Hoeflich et al., 2004), as well as play roles in pathologic situations such as primary pulmonary hypertension (PPH), where the BMPRII cytoplasmic tail is truncated (Foletta et al., 2003).

1.4.2 Modulation Of BMP Signalling

Extracellular control of BMP signalling was first studied during embryogenesis, where the process of morphogenesis (specification of various cell types within the body) was dependent on the local concentration of ligands, which was in part controlled by the presence of secreted extracellular antagonists. Noggin, a cysteine-knot BMP-specific antagonist, will antagonize BMP2, 4 and 7 signals, with a higher affinity for BMP2 and 4 (Balemans and Van Hul, 2002). Structure analysis of Noggin bound to BMP7 showed that Noggin dimers bind to regions within the BMP7 molecule that normally interact with

the type I and type II BMP receptors, effectively blocking the physical contact of BMPs with their receptors (Groppe et al., 2002). Noggin thus competes with the BMP receptors for binding of the BMP ligands. Like Noggin, Chordin is also a secreted BMP-specific antagonist with affinities to BMP2, 4, 6, and 7 and plays an important role in the formation of the dorsal-ventral axis in vertebrates. Its mechanism of action is similar to that of Noggin, where it binds BMP ligands and prevents their interaction with BMP receptors (Groppe et al., 2002). Follistatin was originally isolated from ovarian fluid through its ability to suppress FSH secretion from the pituitary (Balemans and Van Hul, 2002). Although Follistatin has the highest affinity for Activin (Iemura et al., 1998), it also has been shown to antagonize BMP signalling activity through a different mechanism than Noggin or Chordin, involving the binding of Follistatin to regions other than the BMP receptor-interacting regions. This direct binding of Follistatin to BMPs forms a trimeric complex that can still associate with the BMP receptors, but is unable to signal (Iemura et al., 1998). Gremlin, another BMP antagonist belonging to the DAN/Cerberus family of extracellular inhibitors, acts in a similar fashion as Noggin and Chordin (Balemans and Van Hul, 2002), suggesting that these extracellular antagonists may have complementary and/or overlapping functions in vertebrate development. Indeed, knockout studies show that single knockout *noggin* and *chordin* mice did not exhibit any developmental defects, however double *noggin/chordin* knockouts displayed a number of defects, indicating that these modulators have overlapping roles (Bachiller et al., 2000).

BMP signals can also be modulated at the cell membrane level through the presence of membrane-bound regulators. BMP and Activin membrane-bound inhibitor

(BAMBI) was first cloned and characterized in 1999, and acts as an inhibitor of BMP, Activin and TGFß signalling (Onichtchouk et al., 1999). This pseudoreceptor is similar in structure to the type I receptors, but lacks an intracellular kinase domain. Its inhibitory actions are mediated through its intracellular domain, which resembles the homodimerization domain of the type I TGFß superfamily receptors. BAMBI will associate with type I receptors through this domain, effectively inhibiting the formation of complexes with active type I and type II receptors and suppressing signalling activity (Onichtchouk et al., 1999). Conversely, BMP signals have been shown to be enhanced by a novel BMP co-receptor called DRAGON, a glycosylphosphatidylinositol-anchored member of the repulsive guidance molecule family (Samad et al., 2005). This co-receptor has been shown to bind directly to BMP2 or 4, but not BMP7 or any of the other TGFß superfamily ligands. DRAGON associates directly with the type I and II BMP receptors, and its actions are reliant on ligand interaction, as the BMP inhibitor Noggin was shown to reduce the potentiating action of DRAGON in the *Xenopus* embryo. Furthermore, DRAGON has also been shown to be expressed in reproductive tissues including the ovary, and enhance the actions of BMP2 and 4 signalling in reproductive cell lines through BRE-responsive promoter experiments (Xia et al., 2005). Although the mechanism of how DRAGON potentiates BMP2 and 4 signalling is still under investigation, it is thought that DRAGON enhances BMP signalling through binding BMP ligands and stabilizing them with their receptors at the cell membrane within lipid raft structures, facilitating their endocytosis and signalling (Xia et al., 2005).

Intracellular mediators also control BMP signals. As previously mentioned, BMPs negatively regulate their signalling through the expression of I-Smads 6 and 7,

which are direct target genes of BMP signalling (Balemans and Van Hul, 2002). Smad 6 preferentially inhibits BMP signalling, while Smad 7 inhibits TGFß, Activin and BMP signalling (Miyazono et al., 2005). The I-Smads exert their actions through multiple mechanisms, including physical interaction with type II receptor-activated type I receptors, prevention of R-Smad/co-Smad complex formation through interaction of I-Smads with activated R-Smads, and inhibition of Smad-dependent target gene transcription (Miyazono et al., 2005). Smad ubiquitination regulatory factors 1 and 2 (Smurf 1 and 2) are members of the HECT type E3 ubiquitin ligases, and these proteins have been shown to physically associate with and degrade Smads. The WW motif of Smurfs interacts with the PY motif of the Smad linker regions and targets these Smads for ubiquitin-dependent degradation. Smurfs can also associate with I-Smads and induce I-Smad export from the nucleus to the cytoplasm and interact with type I receptors, targeting them for degradation. This leads to down-regulation of the number of cell-surface receptors, and suppression of TGFß superfamily signalling (Miyazono et al., 2005). Smurf 1 has been shown to bind to Smad 6 in the nucleus and the resulting complex was exported to the cytoplasm to target the degradation of type I BMP receptors (Murakami et al., 2003). Furthermore, the BMP type II receptor interacting protein Trb3 can target Smurf 1 for degradation upon BMP signalling, indicating that BMP ligand/receptor interaction can exercise control over Smurf 1 activity and stabilize BMP signalling through the Smad pathway (Chan et al., 2007). Ski and SnoN are protooncogenes that act as TGFß signalling co-repressors. Although they have been extensively studied as Smad 2/3 inhibitors, they can associate with Smad 4 and repress BMP signalling (Liu et al., 2001). Transducer of erbB2 (Tob) is a member of the Tob/B-

cell translocation gene (BTG) family of anti-proliferative proteins that also regulates BMP signalling through its association with Smad 6. Tob participates in stabilizing Smad 6 with type I receptors at the plasma membrane, effectively inhibiting BMP signalling (Yoshida et al., 2003).

Smads 2 and 3 are recruited to the activated TGFß/Activin receptor complex through association with a Smad anchor for receptor activation or SARA (Tsukazaki et al., 1998). Until recently, no such molecule had been identified for the BMP-specific Smads 1, 5 and 8. Shi and colleagues have recently identified that the FYVE-domain protein endofin (endosome associated FYVE domain protein) functions as a Smad anchor for receptor activation in the BMP pathway (Shi et al., 2007). They show that endofin associates with unphosphorylated Smad 1 through its Smad binding domain, and with the BMP type I receptors ALK3 and 6 (Murphy, 2007), and can also regulate the expression of BMP-dependent genes. This interaction has been shown to occur in the early endocytic compartment, suggesting that Smad 1 phosphorylation occurs in the early endosome after internalization of the ligand-receptor complex. This is contrary to the findings of Hartung *et al*, who show that Smad 1 phosphorylation occurs outside the endosome, but that internalization is required for continuation of the BMP signalling cascade (Hartung et al., 2006). These findings indicate that although a tremendous amount of information is being uncovered regarding the regulation of BMP signalling complexes, a great deal more research is needed to clarify signalling events at the cell membrane.

Many of the TGFß superfamily signalling receptors and Smads rely on phosphorylation in order to become activated. The duration of protein phosphorylation,

and thus of cellular signalling in many cell systems is tightly regulated by the action of protein phosphatases. A number of studies have identified phosphatases that are involved in dephosphorylating Smad 1, including pyruvate dehydrogenase phosphatase (PDP) (Chen et al., 2006a), small C-terminal phosphatases (SCP 1-3) (Knockaert et al., 2006), and protein phosphatase 2C alpha (PP2Cα) (Duan et al., 2006). However, Duan and colleagues also show conflicting results in that PDP and SCPs had no effect on Smad 1 phosphorylation. Clearly further studies will elucidate the role of each of these phosphatases in the regulation of BMP-specific Smad phosphorylation.

1.4.3 BMP Signalling In The OSE

During the estrous cycle, BMP2, 3b, 4, 6, 7 and 15 have been shown to play roles in follicle maturation and atresia (Erickson and Shimasaki, 2003), highlighting the role of BMP signalling in normal ovarian function. Of particular interest is the finding that BMPs 3b, and 6 are constitutively expressed in the OSE throughout the estrous cycle, but that BMP4 expression is induced immediately following ovulation only in the OSE cells surrounding the ruptured follicle (Erickson and Shimasaki, 2003). This observation suggests that BMP4 expression is tightly regulated during the ovulatory process, and that BMP4 may participate in the repair of the OSE following ovulation. These results also suggest that since tightly regulated BMP4 expression is likely to participate in the cyclic repair of the OSE following ovulation, a dysregulated BMP4 expression or activity may not only alter ovulatory repair, but may also induce changes in the biology of OSE cells that could lead to pre-neoplastic progression. Support for this notion came from observations in our laboratory that primary human normal OSE and OvCa cells possess an autocrine BMP signalling pathway, where these cells express components of the BMP

signalling pathway and also respond to BMP stimulation (Shepherd and Nachtigal, 2003). Primary normal OSE and OvCa cells were shown to express mRNA for the BMP type I (ALK3 and ALK6) and II (BMPRII) receptors, Smads 1, 5 and 8, as well as for BMP4 and 6. BMP2 was only expressed in immortalized OSE cells and OvCa cells, but BMP7 was not expressed in any of the OSE, OvCa or OvCa cell lines tested (Shepherd and Nachtigal, 2003). Interestingly, a crucial difference between the responses of normal OSE cells and OvCa cells was identified. In response to BMP4 stimulation, *Id1* and *Id3* target gene expression was upregulated 2 to 3-fold in normal OSE cells, as opposed to 10 to 15-fold in OvCa cells, suggesting that OvCa cells have acquired an enhanced sensitivity to the effects of BMP4 stimulation, and that this altered signalling may alter the repair capabilities of the OSE following ovulation.

1.4.4 BMP Signalling In Human Disease Processes

Given the fact that BMP signalling is involved in the homeostasis of many adult tissues, disruption of these signalling pathways has been implicated in many human diseases. Mutations in BMP receptors are found in many autosomal dominant inherited disorders. BMPRII mutations are found in PPH patients, ALK2 mutations in fibrodysplasia ossificans progressiva (FOP), ALK3 mutations in juvenile polyposis syndrome (JPS), and ALK6 mutations in brachydactyly type A2 (Lane et al., 2000; Lehmann et al., 2006; Shore et al., 2006; Waite and Eng, 2003). The deregulation of BMP signalling has also been found in many cancer types. BMP4, 6 and 7 are expressed in prostate cancer cells with known skeletal metastases (Hamdy et al., 1997; Masuda et al., 2003). Furthermore, overexpression of Noggin in prostate cancer cells inhibited the growth of osteolytic prostate cancer lesions (Feeley et al., 2006), suggesting that BMP

signalling is actively involved in prostate cancer metastases. BMP2, 4 and 5 expression have been observed in high-risk and metastatic lesions of the oral epithelium (Jin et al., 2001). BMP4 has been implicated in the metastasis of colorectal cancer cells where it was shown to be overexpressed in advanced stages of colorectal cancer (Deng et al., 2007). BMP4 overexpression in colorectal cancer cell lines increased their motility and invasion, implicating this signalling molecule as a player in colorectal cancer metastasis (Deng et al., 2007). BMP2 has been implicated in the metastasis of breast cancer cell lines, where cells migrate towards a source of BMP2, and when xenografted into mice, BMP2-expressing MCF-7 tumors showed enhanced tumor formation and vasculature (Clement et al., 2005).

Our group has shown that BMP4 signalling may have a role to play in OvCa cell metastasis. As previously mentioned, primary human normal OSE and OvCa cells possess an intact BMP signalling pathway (Shepherd and Nachtigal, 2003). Responses in terms of target gene expression are enhanced in OvCa cells versus normal OSE cells, suggesting that OvCa cells are more sensitive to the effects of BMP4 signalling. Additionally, we have shown that BMP4 treatment of OvCa cells induces enhanced motility, adhesion, invasion capabilities, as well as an EMT response and Rho-GTPase activation, which is characteristic of many highly metastatic cancer cells (Theriault et al., 2007). Interestingly, normal OSE cells do not alter their motility in response to BMP4, highlighting another important difference in the responses between normal OSE and OvCa cells. Taken together, these results point to BMP4 signalling as a player in OvCa cell metastasis.

1.5 Transforming Growth Factor Beta

TGFß, as the name implies, was first identified as a component of "sarcoma growth factor" secreted from Moloney sarcoma virus-infected 3T3 cells that could mediate transformation of non-neoplastic rat kidney NRK and murine AKR-2B fibroblasts (de Larco and Todaro, 1978). Since then, TGFß has been extensively studied, and implicated in almost all aspects of developmental and adult biological processes including cell growth, apoptosis, differentiation, migration, angiogenesis and immunity (Massague et al., 2000). This molecule has been involved in many disease processes as well as cancer cell pathogenesis, but the cellular responses to TGFß can be variable depending on the cell type and extracellular environment. TGFß can cause epithelial cells to undergo growth arrest and apoptosis, suggesting that it plays a tumor-suppressor role, while it can induce EMT in fibroblasts and increase their proliferation, promoting tumorigenesis and fibrosis (Rahimi and Leof, 2007).

1.5.1 TGFß Signalling Pathways

TGFß ligands also exist as dimers linked by a disulfide bridge, possessing the characteristic cysteine knot structure. Three isoforms of TGFß (1-3) are present within mammals. TGFß is initially synthesized as a precursor protein which is processed into a latent form composed of the mature TGFß molecule non-covalently bound to the amino-terminal portion of the proprotein called latency-associated peptide (LAP) (Munger et al., 1997) and exerting control over TGFß ligand actions even before associating with receptors (Figure 1-6). After secretion, the latent TGFß can then be activated by a number of proteolytic enzymes, including plasmin, urokinase (or urokinase-like plasminogen activator), as well as MMPs 2 and 9. Other proteins present in the

extracellular environment such as thrombospondin-1 and integrin receptors can also bind to latent TGFß and participate in its activation (Prud'homme, 2007). It is important to note that LAP-TGFß is usually secreted as a large latent complex consisting of LAP-TGFß covalently bound to a latent TGFß binding protein (LTBP) (Hyytiainen et al., 2004). LTBPs are proteins that compose the ECM, and have been shown to target latent TGFß for incorporation into ECM, thus affecting the immediate availability of the protein. The ECM can therefore act as a reservoir of TGFß molecules that can be activated upon matrix remodeling. In this way, the extracellular environment controls remodeling functions by providing a source of growth factors to modulate normal tissue homeostatic processes such as wound healing, but conversely can also play a role in pathogenic processes such as in fibrosis or cancer cell growth and metastasis (Rahimi and Leof, 2007). The activated TGFß molecule can then associate with 3 types of TGFß receptors, type I (ALK1, ALK2 or ALK5), type II (TβRII), and type III (betaglycan). Betaglycan acts as a co-receptor, binding to all isoforms of TGFß with high affinity, and mediating the binding of TGFß to the type II receptor. Unlike BMP signalling, TGFß isoforms have higher affinity for their respective type II than their type I receptors (Shi and Massague, 2003). Once the type II receptor binds ligand, the constitutively activated receptors will phosphorylate the type I receptors at its GS domain, forming a heterotetrameric complex of type I and type II receptors. In the canonical TGFß pathway, the activated type I receptor (the predominant one being ALK5) will go on to phosphorylate and activate R-Smads 2 and/or 3. These activated Smads will combine with the common Smad 4, and translocate to the nucleus to affect target gene expression. In endothelial cells, endoglin instead of betaglycan, has been shown to act like a type III

TGFß receptor and promote TGFß signalling though the activation of ALK1 (Lebrin et al., 2004) and subsequent activation of Smads 1, 5, and 8. In the same manner, ALK2 has been shown to associate with TßRII in cardiovascular cells during development and activate Smads 1, 5 and 8 (Shi and Massague, 2003), indicating that the BMP-specific Smads are not necessarily restricted from being activated solely through the phosphorylation activity of BMP type I receptors.

As in the case of BMP signalling, TGFß ligands and receptors associate at the cell membrane to form active signalling complexes through 2 types of membrane compartments: CCPs and cholesterol-rich caveolae or lipid rafts (Di Guglielmo et al., 2003). Although TGFß receptors have been shown to constitutively traffic into different endocytic pathways without the presence of ligand, the type of membrane compartment in which the ligand-bound receptor complexes are located will dictate the type of signalling that occurs. Receptors located within CCPs can signal through R-Smads, while receptors located within caveolae are destined for degradation by ubiquitination (Di Guglielmo et al., 2003). Evidence for this mode of signal transduction arises from the finding that the localization of SARA was shown in CCPs as part of the early endosome; this localization would enable SARA to recruit Smads 2 and 3 to the activated type I receptor and become phosphorylated (Di Guglielmo et al., 2003). These findings are in agreement with those of Shi and colleagues, who determined that endofin, a novel Smad anchoring protein for the BMP family of signalling molecules, is also located within the early endosome and mediates BMP-specific Smad phosphorylation (Shi et al., 2007). Conversely, Smad 7 and Smurf 2, which are negative regulators of TGFß signalling as part of the ubiquitin-ligase complex, were localized within caveolae, indicating that the

receptor complexes located within these membrane compartments are destined for degradation. The evidence that in certain cell types SARA binds directly to type I receptors, and that blocking CCP-dependent endocytosis does not abolish TGFß signalling suggests that these two endocytic pathways may not be essential in transducing TGFß signalling (Lu and al., 2002). These results highlight the complex nature of TGFß signalling, which may be varied between cell types.

Many target genes are regulated by direct TGFß/Smad-dependent signal transduction pathways. In the case of TGFß-induced growth inhibition in epithelial cells, TGFß activates the transcription of cell cycle inhibitors such as $p21^{Cip1/WAF1}$, and $p15^{Ink4b}$ (Rahimi and Leof, 2007). TGFß inhibits the transcription of the pro-growth transcription factor c-myc, as well as Id proteins. TGFß signalling can also induce pro-apoptotic responses by upregulating the FAS receptor through Smad 3-dependent mechanisms, as well as inducing the transcription of the death associated protein kinase (DAPK) and regulating the expression of Bcl-2 family members (Rahimi and Leof, 2007). Additionally, TGFß signalling can induce apoptosis of haematopoietic cells by changing intracellular phospholipid pools through the direct target gene induction of the inositol phosphatase Src homology 2 domain-containing 5' inositol phosphatase (SHIP) (Valderrama-Carvajal et al., 2002). SHIP induction by TGFß was found to be Smad-dependent, and was followed by inhibition Akt/protein kinase B (PKB) phosphorylation and cell survival (Valderrama-Carvajal et al., 2002). During EMT progression, TGFß enhances the Smad-dependent transcription of Snail and Slug, two transcription factors involved in repressing E-cadherin expression, which is a crucial step in the progression of EMT (Cho et al., 2007). Furthermore, it has been shown that in prostate cancer cells,

Smad 7 mediates the activation of Cdc42, an important cytoskeletal remodeling protein in EMT progression (Edlund et al., 2004). Smad-independent pathways of TGFß signal transduction have also been extensively documented. The best characterized of these pathways include the activation of MAP kinase members (Jun-N terminal kinase) JNK and p38 in the induction of apoptosis. ALK5 can interact with Par6, a tight junction protein, and cause tight junction dissolution, which is also a hallmark of EMT progression. Conversely, TßRII can induce Smad-independent, ERK- and phosphoinositide -3 kinase (PI3K)-dependent EMT through the upregulation of Snail, revealing the complexity of TGFß signalling in the induction of specific behaviours. Whether these signalling mechanisms are cell and/or context-dependent remain to be determined, however these findings indicate that there may be some redundancy in the responses of cells to TGFß.

1.5.2 Modulation Of TGFβ Signalling

Extracellular control of TGFß signalling first starts at secretion. As previously mentioned, LAP-TGFß is secreted covalently bound to LBTPs, where they are inserted into the surrounding ECM. TGFß will be released from the ECM through proteases such as MMPs during matrix remodeling, where further proteolysis will activate TGFß through the removal of LAP. As TGFß has been shown to affect a multitude of cellular processes, this mode of secretion and storage in the extracellular environment controls the amount of active TGFß present to induce signalling. Other TGFß binding proteins can control the access of the ligand to the receptors, including the small proteoglycan decorin, and the circulating protein α-2 macroglobulin, which will bind free TGFß (Shi and Massague, 2003).

Cell membrane mediators include betaglycan or endoglin, the type III TGFß receptors, which act as co-receptors to facilitate ligand access to type II receptors (Lebrin et al., 2004; Prud'homme, 2007; Shi and Massague, 2003). In an opposite fashion, BAMBI will negatively regulate TGFß signalling by acting as a pseudoreceptor and sequestering the type I receptors from interacting with the type II receptors, with the end result of signalling suppression (Onichtchouk et al., 1999). Intracellular mediators include the I-Smad, Smad 7, which is a direct target gene of TGFß signalling. Smad 7 will bind to the activated receptors in competition with Smads 2 and 3 (Shi and Massague, 2003). Additionally, Smad 7 interaction will lead to the ubiquitination of Smads by the Smurfs, which occurs in caveolae/lipid raft-rich membrane compartments, while SARA will facilitate Smad interaction with type I and type II receptor complexes located within CCP/early endosomal compartments (Di Guglielmo et al., 2003). Another mediator of TGFß signalling includes Trap-1-like protein (TLP), which has been shown to act as a molecular switch regulating the balance of Smad 2- versus Smad 3-dependent signalling (Felici et al., 2003). TLP interacts with the type II receptors in the absence of signalling, and upon ligand binding, directs activated receptors to Smad 3-containing endocytic domains and facilitate the formation of Smad 3/4 complexes, while this interaction does not occur in the case of Smad 2-dependent signalling. c-Ski and SnoN are highly conserved members of the Ski family of protooncoproteins that are negative regulators of Smad-dependent transcription. They antagonize TGFß signalling through competing with the R-Smads for Smad 4 binding, preventing the formation of a functional signalling complex between R-Smads and Smad 4 (Shi and Massague, 2003). Furthermore, c-Ski and SnoN will inhibit the Smads from binding to the transcriptional

activator E1A binding protein p300 (p300)/CREB binding protein (CBP), and recruit nuclear co-repressors and the histone deacetylase complex which will inhibit target gene transcription (Shi and Massague, 2003). Menin, a product of the multiple endocrine neoplasia type 1 (MEN1) tumor suppressor gene, is a transcriptional regulator that has been shown to facilitate TGFß-induced target gene expression by specifically interacting with Smad 3 through its MH2 domain. Targeted antisense disruption of menin transcription antagonized the growth-inhibitory effects of TGFß signalling in pituitary cells, revealing the essential role of this molecule in modulating Smad 3-dependent DNA binding and transcriptional activation (Kaji et al., 2001). G-coupled receptor kinase 2 (GRK2) was recently identified as a TGFß and Activin target gene that acts as a negative regulator of TGFß- and Activin-induced target gene expression (Ho et al., 2005). The mechanism by which GRK2 exerts its effects is through the phosphorylation of Smad proteins within their linker region, preventing Smad activation by the TGFß and Activin type I receptors and resulting in the inhibition of nuclear translocation and target gene expression (Ho et al., 2005).

Protein phosphatases will also regulate the activation status of Smads 2 and 3 and terminate TGFß-induced signalling. The phosphatase PPM1A/PP2Cα has been the only phosphatase identified to date to directly act upon Smad 2 and 3 and affect their phosphorylation status in the nucleus to inhibit TGFß signalling (Lin et al., 2006b).

1.5.3 TGFß Signalling In The OSE

TGFß is a well-known inhibitor of epithelial cell proliferation. Several studies have demonstrated that OSE proliferation is inhibited *in vitro* by TGFß (Wong and Leung, 2007). Furthermore, TGFß has been shown to inhibit the growth of OSE that has

been stimulated to proliferate by TGFα or EGF, as well as decrease OSE cellular invasive capabilities (Auersperg et al., 2001; Rodriguez et al., 2001). Normal OSE and the underlying stromal cells in the ovary cells express ligands and receptors for TGFß signalling, indicating that the components are available for effective autocrine and paracrine signalling to occur (Nilsson and Skinner, 2002). It is thought that the growth inhibitory function of TGFß in the OSE may play a role in preventing the over-proliferation of the cells during the repair processes initiated following ovulation, and may therefore act as a regulating factor in OSE repair. However in OvCa cells, TGFß signalling has been shown to have antagonistic roles as compared to normal OSE at later stages of tumor progression, indicating that during the process of OvCa formation and metastasis, OvCa cells may adopt characteristics that allow them to escape growth inhibition and become highly invasive. These characteristics will be discussed below.

1.5.4 TGFß Signalling In Human Disease Processes

Many mutations in TGFß receptors account for a number of hereditary disorders. Camurati-Engelmann disease (CED) is caused by a mutation in the TGFß1-encoding gene which increases ligand signalling, leading to a bone disorder characterized by inflammation of the limbs and skull. Mutations within the TßRII gene can cause Marfan Syndrome (MFS), which is a result of enhanced TGFß signalling in the cardiovascular, skeletal and ocular systems and causes aortic aneurysms, dislocation of the ocular lens, and disproportionately long limbs. ALK1 mutations account for the autosomal-dominant disease Hereditary hemorrhagic telangiectasia (HHT), which is characterized by small vascular lesions affecting most organs (Harradine and Akhurst, 2006).

Mutations within TGFß signalling molecules have also been shown to account for cancer predisposition syndromes, such as JPS and Cowden-like syndrome (CS). These are both autosomal-dominant polyposis syndromes that increase a patient's risk for developing gastrointestinal malignancies, including colorectal cancer. Both possess mutations in Smad 4, which will compromise the ability of most TGFß superfamily members to signal properly, as this Smad is common to all superfamily ligands (Harradine and Akhurst, 2006). Mutations in TßRI have been identified in many cancers, including chronic lymphocytic leukemia, prostate cancer and glioblastoma, while mutations in TßRII have been identified in gastric, colon, endometrial, colorectal, lung and ovarian cancers. Smad 2 has also been found mutated in colorectal and lung cancers, suggesting that dysregulation of the TGFß signalling pathway is involved in cancer progression (Wang et al., 2000). Most of the characterized mutations are inactivating, indicating that the changes in TGFß signalling responsiveness could be responsible for most of the TGFß resistance of cancer cells to growth inhibition during later stages of the disease (Bierie and Moses, 2006). Interestingly, mutations in TßRI, TßRII and Smad2 have been found in OvCa, also implicating this signalling pathway in OvCa carcinogenesis (Wang et al., 2000).

TGFß signalling has been extensively studied as a pathway that controls the progression of many cancers. TGFß appears to have a dualistic role in cancer development depending on the cell type and the extracellular influence to which the cancer cells are exposed. It is believed that in initial cancer stages as with normal epithelial cells, TGFß signalling acts as a tumor suppressor where it induces growth inhibition. This has been shown in OvCa cells, where TGFß can inhibit early-stage OvCa

cell proliferation through the downregulation of the anti-apoptotic factor Bcl-2 (Chow et al., 1996). At later stages of OvCa progression however, TGFß signalling has been shown to stimulate tumor growth and metastasis (Nilsson and Skinner, 2002). TGFß was found to be upregulated in some OvCa tumors, while TGFß receptor levels were increased in others, indicating that OvCa cells could possibly show altered signalling capabilities (Nilsson and Skinner, 2002). In fact, during tumor progression, tumor cells frequently lose the growth-inhibitory response to TGFß, and this is associated with an increased expression of TGFß in the microenvironment (Bierie and Moses, 2006). This escape from TGFß-induced growth inhibition could be due to amplification or overexpression of the cell cycle stimulator c-myc, or inactivation of the cell-cycle inhibitors p15 or p21. Mutations present within TGFß receptors in cancer cells could also contribute to the escape from growth inhibition (Levy and Hill, 2006). Tumor progression is also associated with the development of EMT responses, where the tumor cells loose intercellular contacts, remodel their cytoskeleton into a mesenchymal-like migratory cell phenotype and increase MMP production, which will all promote cellular invasion and metastasis behaviours. Once the cancer cells acquire the capacity to evade the inhibitory effects of TGFß, there is a selective advantage for these cells to now produce and/or activate more TGFß and further promote this carcinogenic process, by remodeling the ECM and the stroma surrounding the tumor cells, releasing and activating more TGFß stored within the ECM which stimulates the stromal cells to proliferate and induce more ECM production, as well as promote tumor vascularization and the production of factors to suppress the immune recognition of the cancer cells. Therefore, TGFß produced from the tumor cells can act in the local microenvironment to provide a

positive signalling feedback loop, producing a favorable niche for tumor growth, invasion and metastasis (Leivonen and Kahari, 2007). Although mutation in TGFß signalling components can occur in cancer cells that could explain the development of resistance to growth inhibition, complete abrogation of the signalling pathway is unlikely due to the fact that many aggressive cancers still possess the ability to respond to TGFß in a tumor-promoting manner. How do cancer cells therefore make this switch in responsiveness to TGFß? The answer may lie in the amount of TGFß signalling present. Evidence shows that point mutations present in TGFß receptors that reduce their activity cause TGFß resistance to growth inhibition in these cells, but do not alter certain target genes involved in TGFß-induced cellular migration, such as plasminogen activator inhibitor 1 (PAI-1) (Levy and Hill, 2006). Further investigation into the threshold of TGFß activity required to produce these dualistic effects requires further investigation.

Although many reports show that TGFß signalling promotes tumor growth in OvCa cells, other reports show that TGFß actually inhibits growth to a much lesser degree than with earlier stage cancers (Nilsson and Skinner, 2002). In fact, it has been shown in our laboratory that TGFß is growth-inhibitory to primary OvCa cells isolated from late-stage patients, and that this growth inhibition can be counteracted by treatment with EGF. This scenario would suggest that instead of promoting OvCa tumorigenesis, TGFß signalling may retain its tumor suppressive activity in OvCa cells throughout their progression, and that other signalling molecules may be responsible for their proliferative and invasive effects (Dunfield and Nachtigal, 2003). As previously mentioned, this type of alterative behaviour could be in part due to the amount of TGFß signalling present in

these OvCa cells (Levy and Hill, 2006); however, the mechanisms behind this variability in TGFß responses remain to be determined.

1.6 Activin

Since Activins were first isolated from the gonads as regulators of FSH secretion (Ling et al., 1986), they have been shown to exert effects in many physiological processes during embryogenesis and adult physiology including cellular proliferation, differentiation, apoptosis and homeostasis. Activin plays a crucial role in ovarian function through the regulation of FSH secretion, and a functional Activin signalling pathway has been identified in normal OSE and OvCa cells (Ito et al., 2000). Although Activin and TGFß share many similarities in signalling pathways and cellular effects, some differences have been observed with respect to cellular responses during normal tissue homeostasis, as well as in pathogenic situations.

1.6.1 Activin Signalling Pathways

Activins are dimeric proteins that are composed of different arrangements of ß subunits (Activin A [ßA ßA], Activin AB [ßA ßB], or Activin B [ßB ßB]), which are linked by a disulfide bridge. Other Activins have been identified (C, D and E), however little is known about their biological significance (Roberston et al., 2004). As the other members of the TGFß superfamily of ligands, Activins are secreted as proproteins that need to be proteolytically processed at their amino terminus in order to become biologically active. This processing occurs within the cell, and Activins are secreted in their processed, biologically active form (Harrison et al., 2004) (Figure 1-7). The active ligand can associate with type I (ALK4, ALK2 or ALK7) and type II (ActRII or ActRIIB) receptors to transduce their signals. As with TGFß, Activins have a high

affinity for the type II receptors and do not stably interact with the type I receptors. Ligands will first associate with the type II receptors, allowing for the type I receptors to bind. These interactions will form a large heterotetrameric complex of type I and type II receptors, where the constitutively active type II receptors can phosphorylate and activate the type I receptors (Shi and Massague, 2003). In the canonical Smad signal transduction pathway, Activin signalling will activate Smads 2 and 3, associate with Smad 4, and translocate to the nucleus to affect target gene expression. Based on the similar Smad activation pathways, similarities in receptor structure and intracellular regulators of signalling, Activins have often been grouped in the same family with TGFß ligands, and it is believed that Activin receptor dynamics at the cell membrane and association with distinct cellular membrane compartments are similar to those of TGFß signalling (Di Guglielmo et al., 2003). Activin receptor-interacting proteins (ARIPs 1 and 2) have been identified to interact with the type II Activin receptors, and cause receptor internalization and effective decrease in signalling (Tsuchida et al., 2004); however whether these proteins are associated with I-Smads or Smurfs in lipid raft membrane compartments remains to be determined.

After translocation into the nucleus, activated Smads regulate the transcription of target genes by binding to consensus DNA sequences and interacting with transcriptional co-activators and co-repressors. Although Smads 2 and 3 are structurally very similar, they transmit different signals because of their differing ability to bind DNA. The common splice variant of Smad 2 contains a DNA insertion within the DNA binding domain, resulting in a poor DNA binding efficiency as compared with Smad 3 (Shi and Massague, 2003). Smad 2 therefore requires other accessory proteins in order to

effectively activate Smad 2-dependent gene transcription. It is thought that Smad 2 is a transducer of Activin signals based on the findings that Smad 2 is responsible for transducing Activin signals during embryogenesis, while Smad 3 is a TGFß-specific transducer of signals for cellular growth, movement and immune function in adult tissues (Miyazono et al., 2001). However, both Smad 2 and 3 can be activated by TGFß signalling in cultured keratinocytes, but only Smad 3 can by activated by Activin A (Shimizu et al., 1998). Furthermore, Smad 2 and 3 were shown to associate transiently with ALK4 in an inducible type I/II Activin receptor-expressing KAR6 cell line. After this rapid association, Smads 2 and 3 were released into the cytoplasm where they interacted with Smad 4 and mediated Activin-induced gene transcription (Lebrun et al., 1999). Thus the specificity of Smad 2 or 3 signalling in dictating Activin or TGFß-specific signalling responses warrants further investigation.

Activin acts as an inhibitor of cellular proliferation and apoptosis through the upregulation of target genes including the cell cycle inhibitors $p21^{Cip1/WAF1}$, $p15^{Ink4b}$ and $p27^{KIP1}$ (Chen et al., 2006b). As with TGFß, Activin signalling induces apoptosis by altering the phospholipid balance in haematopoietic cells through the Smad-dependent expression of SHIP (Valderrama-Carvajal et al., 2002). Activin is also a player in the inflammatory process, showing pro-inflammatory effects by the upregulation of TNFα, IL-1ß and cyclooxygenase 2 (COX-2), but as opposed to TGFß, also showing anti-inflammatory effects through the inhibition of IL-6 transcription (Jones et al., 2004). GnRH is also a target of Activin signalling, and will control the reproductive axis by stimulating GnRH production and release in the pituitary to affect gonadal secretion of LH and FSH. Activin can also activate the Smad-dependent transcription of Follistatin

and Follistatin-related gene (FLRG), stimulating a negative feedback signalling loop (Bartholin et al., 2002). Activins also affect cellular behaviour through Smad-independent mechanisms. They have been shown to activate MEKK1-JNK in the control of epithelial cell movements, directly increase p38 phosphorylation in the induction of breast cancer cell apoptosis, as well as interact with TGFß-activated kinase 1 (TAK1) to activate the expression of neurogenin 3, a transcription factor essential for pancreatic cell differentiation (Chen et al., 2006b; Cocolakis et al., 2001).

1.6.2 Modulation Of Activin Signalling

Many cell membrane and intracellular modulators of the TGFß signalling pathway also act at controlling Activin signalling, including betaglycan, endoglin, BAMBI, SARA, I-Smads, Smurfs, c-Ski/SnoN and intracellular phosphatases; these have been discussed earlier. Follistatin is a secreted inhibitor that has a higher affinity for Activin than Activin receptors or the other TGFß ligands (Lin et al., 2006a). The binding of Follistatin to Activin is nearly irreversible (Abe et al., 2004). Two molecules of Follistatin usually bind to one Activin dimer, and Follistatin's structure enables it to mimic the type I receptor binding domain, binding to Activin in such a manner as to mask most of the binding epitopes for the type I and type II receptors, and effectively blocking the ligand/receptor interaction and neutralizing Activin-dependent signalling (Lin et al., 2006a). FLRG is a follistatin-related protein, and has similar functions as Follistatin in blocking Activin signalling, but has a different tissue expression pattern than Follistatin, suggesting a complementary antagonistic function of FLRG in non-Follistatin expressing tissues (Lin et al., 2006a). Cripto is a prototypical member of the EGF-Cripto-FRL-Criptic (EGF-CFC) protein family, but does not bind to EGF receptors.

Cripto exists in two forms, a membrane-bound and soluble form; the membrane-bound form acts as a co-receptor for Nodal, another member of the TGFß superfamily of ligands, while the soluble form acts as a growth factor-like signalling molecule. Cripto will inhibit Activin signalling through the binding of Activin and inhibition of its recruitment to the ALK4 receptor (Chen et al., 2006b). The mechanism through which Cripto stimulates Nodal signalling but yet antagonizes Activin signalling is still under investigation. Recent studies have identified ARIPs 1 and 2, which can interact with ActRIIs, and can modulate their levels, thus controlling the level of Activin signalling present in the cell. As previously mentioned ARIP2 can interact with the type II receptors and cause their endocytosis, decreasing levels of free type II receptors to react with ligand (Chen et al., 2006b).

1.6.3 Activin Signalling In The OSE

An intact Activin signalling pathway has been identified in normal OSE cells and OvCa cells (Ito et al., 2000), suggesting that autocrine Activin signalling may regulate OSE repair following ovulation. Indeed, the follicular fluid released from the mature follicle during ovulation flows over the surface of the ovary, and many growth factors, including Activin may provide a stimulus for the proliferation of the OSE cells and initiate the healing process immediately following ovulation (Ito et al., 2000). However, this same group showed later on that Activin treatment had no effect on normal OSE cells in culture, and was growth-inhibitory in preneoplastic OSE (Choi et al., 2001). In contrast, most OvCas secrete Activin *in vitro,* and serum levels of Activin are frequently elevated in women with OvCa (Wong and Leung, 2007), suggesting that an alteration in the cellular responses to Activin signals occurs during OvCa formation.

1.6.4 Activin Signalling In Human Disease Processes

Some rare cases of hereditary developmental disorders have been identified with mutations in the ActRIIB receptor, characterized by left-right axis malformations such as randomization of organ positioning within the body, showing the importance of Activin signalling in embryonic development (Harradine and Akhurst, 2006). Mutations in ActRII have also been identified in 44-90% of colons cancers, 44% of gastric carcinomas, and many pancreatic tumors, all of which decrease Activin signalling capacities (Levy and Hill, 2006). Furthermore, frequent mutations and losses of chromosome 2q where many of the Activin genes are located are found in prostate and ovarian cancers (Risbridger et al., 2001).

Activin acts similarly to TGFß in terms of its dualistic effects on cancer cell proliferation. Activin has been shown to act as a tumor suppressor by inhibiting the growth of tumor cells from prostate, pituitary and breast cancers (Chen et al., 2006b; Cocolakis et al., 2001), as well as being an inhibitor of angiogenesis by decreasing the expression of VEGF receptor 2 in neuroblastoma cells, and inhibiting proteolytic activity, migration and proliferation in endothelial cells (Chen et al., 2006b). By contrast, Activin signalling has been shown to be a tumor promoting factor by stimulating proliferation of multiple OvCa cell lines, including SKOV3, OCC1 and OVCAR-3, as well as mediating high N-cadherin expression on esophageal cancer cells, which was associated with tumor aggressiveness and poor prognosis (Yoshinaga et al., 2004). Activin does not seem to have an effect on normal or preneoplastic OSE, but exogenous Activin is mitogenic in many OvCa cell lines, and serum levels are frequently elevated in women with OvCa (Wong and Leung, 2007). These results suggest that during tumorigenesis OvCa cells

acquire the ability to respond to Activin signalling in a growth-promoting manner. This theory was investigated by Steller and colleagues, who studied the effects of Activin and Inhibin treatment on the proliferation of OvCa cell lines. Inhibin is another member of the TGFß superfamily of ligands that antagonizes the effects of Activin signalling in many endocrine tissues, including the ovary. This group showed that some OvCa cell lines acquired Inhibin resistance, and thus the effects of Activin went unchallenged, resulting in increased proliferative and invasive capabilities in response to Activin signalling (Steller et al., 2005). Therefore the development of Inhibin resistance may play a role in OvCa tumorigenesis.

Contrary to these conclusions, our group has studied the effects of Activin A on primary OvCa cell proliferation and motility, and have shown that Activin is growth-inhibitory and reduces cellular motility in culture (B. Thériault, presented in Chapter 5), suggesting that Activin signalling may be tumor-suppressive. Taken together, these results demonstrate the dichotomous nature of Activin signalling and that, as with TGFß signalling, the effects of Activin signalling on OvCa cells could reveal interesting distinctions depending on the environmental context.

1.7 Hypothesis

The role of BMP signalling in primary human normal OSE and OvCa cells is still poorly understood, however it is suggested that BMP4 may play a role in the repair processes following ovulation, and thus may also have a role to play in OvCa pathogenesis. Although a functional BMP signalling pathway has been identified in normal OSE and OvCa cells, along with the identification of differences in BMP4-induced target gene responsiveness between normal OSE and OvCa cells (Shepherd and

Nachtigal, 2003), functional consequences of BMP4 signalling on normal OSE and OvCa cell biology have yet to be defined. The initial finding from our laboratory that BMP4 treatment of normal OSE and OvCa cells induces a cell spreading morphology suggests that BMP4 signalling is altering cellular components that are responsible for the manifestation of cellular behaviours such as morphology, adhesion, motility as well as invasiveness. My hypothesis is that treatment of primary human OvCa cells with exogenous BMP4 will alter OvCa *in vitro* cellular behaviours. The primary objective of this study was to determine the behavioural effects of BMP4 treatment on OvCa cells through the assessment of cellular morphology, adhesion, motility and invasion. Intracellular signalling molecules that are known to mediate such changes in cellular behaviour were also assessed and potential therapeutic interventions are proposed. The secondary objective of this study was to evaluate the contribution of other TGFß superfamily signalling molecules, such as BMP6, TGFß1 and Activin A to the behaviour of primary OvCa cells. The investigation of the individual contribution of these signalling pathways revealed antagonistic effects between BMP and TGFß/Activin signalling that formed the basis for a proposed model of OvCa tumorigenic spread.

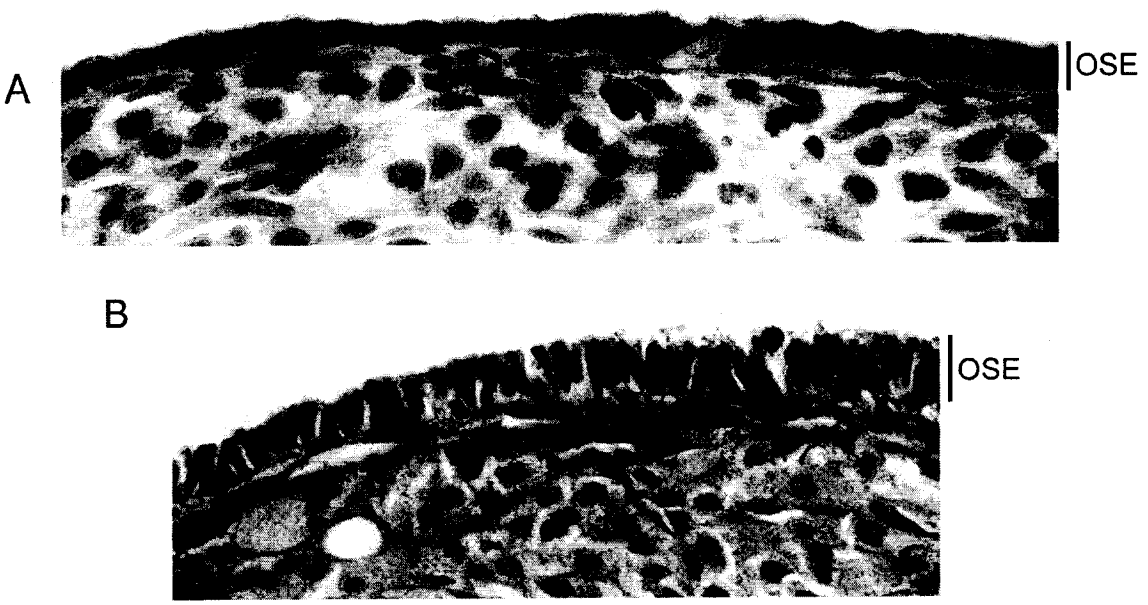


Figure 1-1. Histology of the mouse OSE. Histological sections of mouse OSE depicting the OSE cells existing as a cell monolayer covering the ovarian surface. **(A)** OSE cells are usually squamous-to-cuboidal in shape, but can also exhibit a simple columnar morphology **(B)**. The OSE is separated from the underlying stroma by a semi-permeable basement membrane.

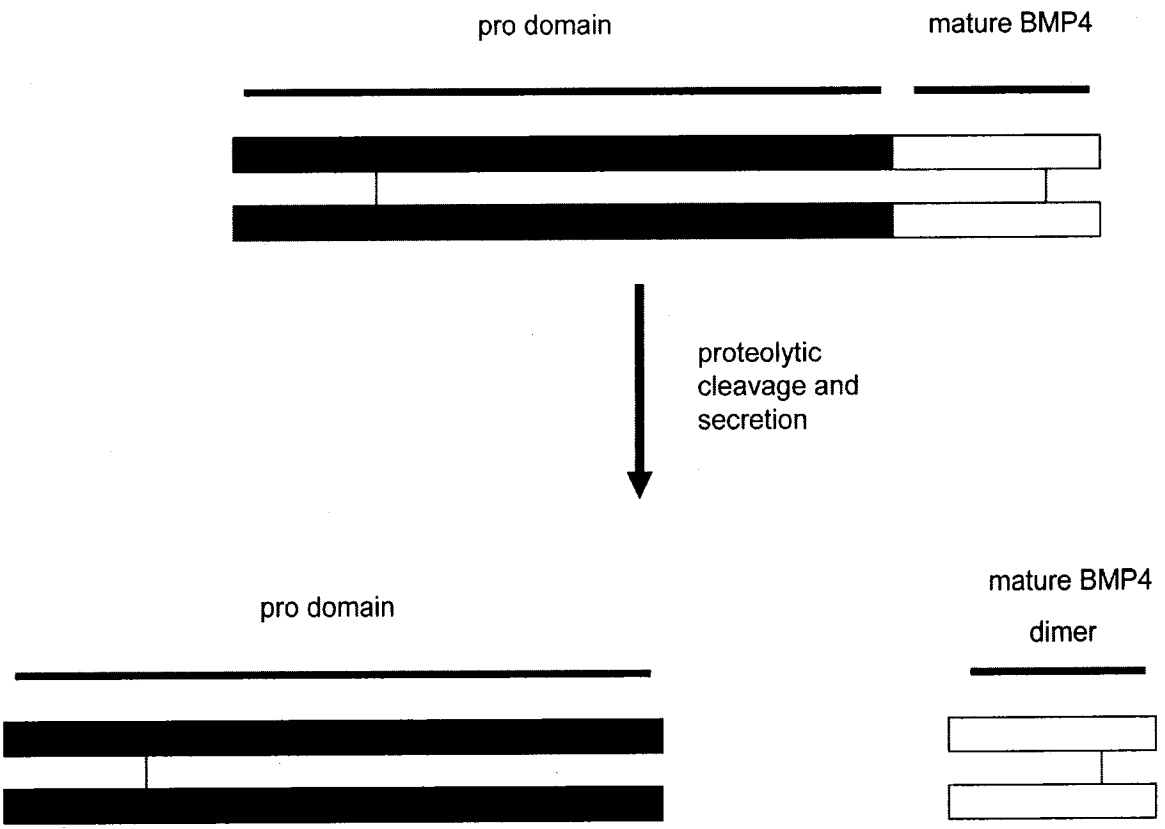


Figure 1-2. Proteolytic cleavage of mature BMP4. BMP4 is synthesized as a dimerized precursor molecule that is proteolytically cleaved prior to secretion. Cleavage results in the formation of a mature BMP4 dimer that can interact with BMP receptors.

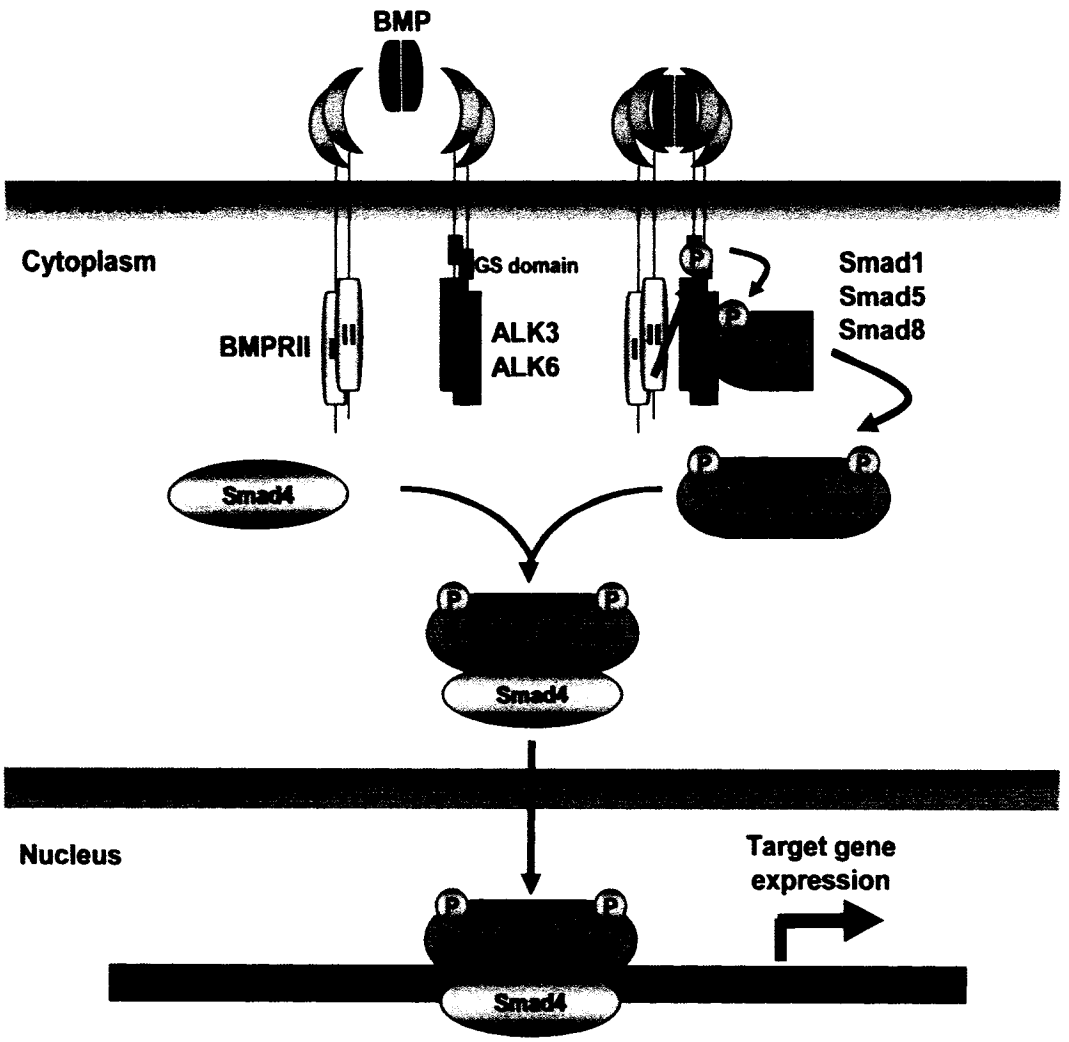


Figure 1-3. Canonical BMP signal transduction pathway. BMP dimers will interact with type I BMP receptors (ALK3 and 6), subsequently recruiting the type II BMP receptors and forming a heterotetrameric signalling complex. The type II receptor, being constitutively activated, will trans-phosphorylate the type I receptor at the GS domain, activating its kinase activity. The activated type I receptor can then go on to phosphorylate and activate R-Smads 1, 5 and 8. These activated Smads can partner with the common Smad 4 and translocate to the nucleus to affect the transcription of target genes.

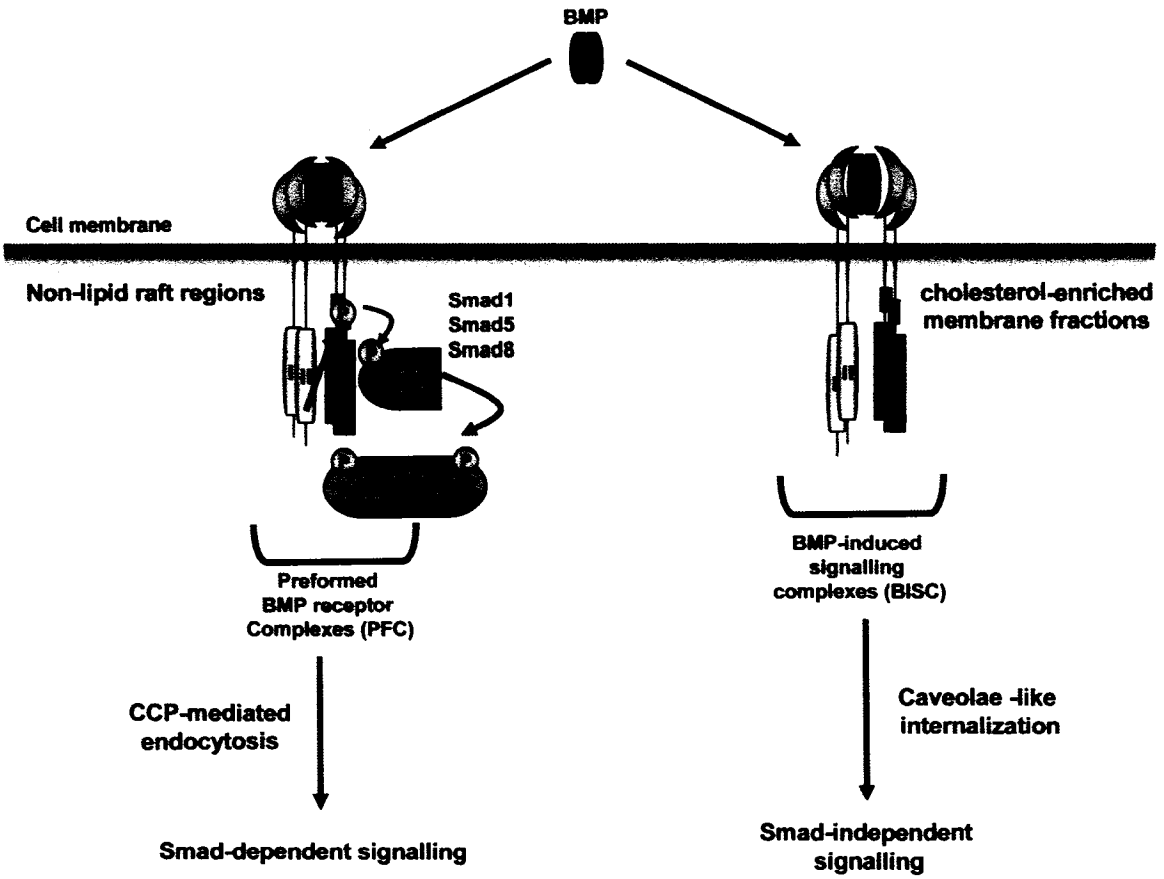


Figure 1-4. Mechanisms of BMP receptor endocytosis and signalling. The membrane localization and endocytic pathway of BMP receptors will dictate the type of intracellular signalling pathways through which receptor signals will be transduced. BMP receptors located in non-lipid raft/clathrin-rich membrane regions are part of preformed BMP receptor complexes (PFC) that undergo Smad phosphorylation at the cell membrane, and are subsequently endocytosed via clathrin-dependent mechanisms to conduct Smad-dependent signalling and target gene expression. BMP receptors located in cholesterol-enriched/caveolae-like membrane fractions are part of BMP-induced signalling complexes (BISC) that will internalize through caveolae-dependent mechanisms and conduct Smad-independent signalling.

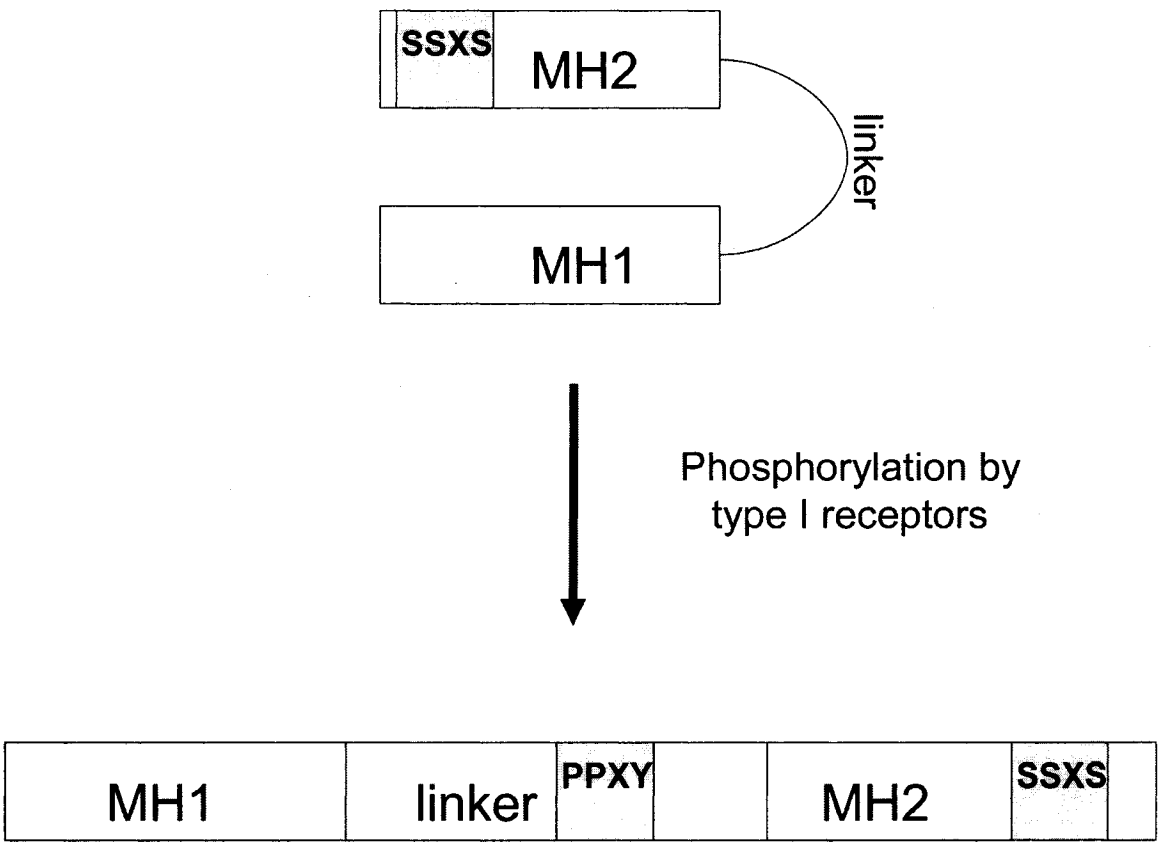


Figure 1-5. Structure of Smad proteins. All Smad proteins contain a highly conserved N-terminal MH1 domain, a linker region, and a C-terminal MH2 domain. The MH1 domain is involved in the binding of specific DNA sequences on target genes. The linker region contains a PPXY motif that can associate with WW domain-containing proteins such as the Smurfs, and regulate Smad activity. The MH2 region is involved in the formation of homo- and heteromeric protein complexes. Smad proteins are usually under autoinhibitory control, where the MH1 and MH2 domains interact. During TGFβ superfamily signalling, activation of the Smad proteins occurs when the activated type I receptor phosphorylates the last 2 serine residues on the MH2 domain's SSXS motif. This allows for the autoinhibitory control to be released, and the R-Smads can associate with the co-Smad4 to complete Smad-dependent signalling.

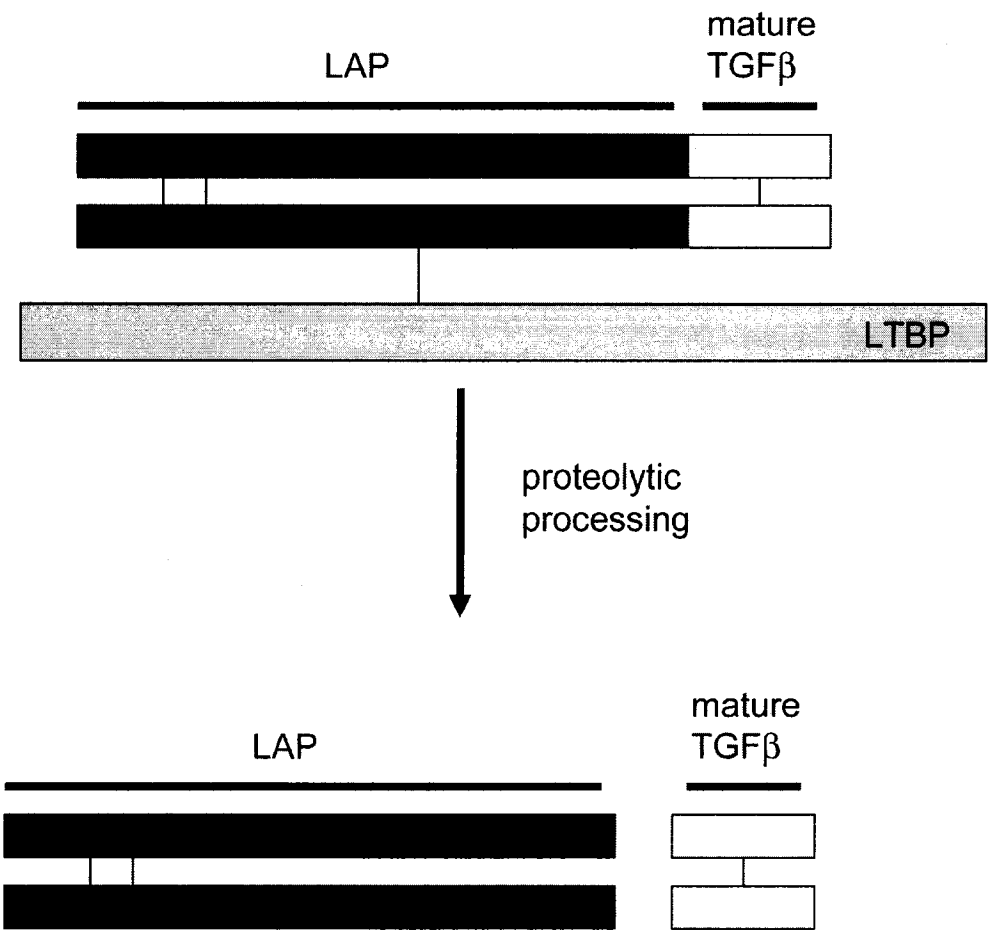


Figure 1-6. Proteolytic cleavage of mature TGFβ. The latent form of TGFβ is secreted as an inactive precursor covalently bound to TGFβ latency-associated peptide (LAP) and latent TGFβ binding protein (LTBP). LTBP will target the latent TGFβ molecule to become incorporated within the ECM. Extracellular proteases can cleave the precursor into the latent TGFβ which is non-covalently associated with LAP and still inactive. Further proteolytic processing will cleave LAP, releasing the activated TGFβ dimer that can interact with TGFβ receptors.

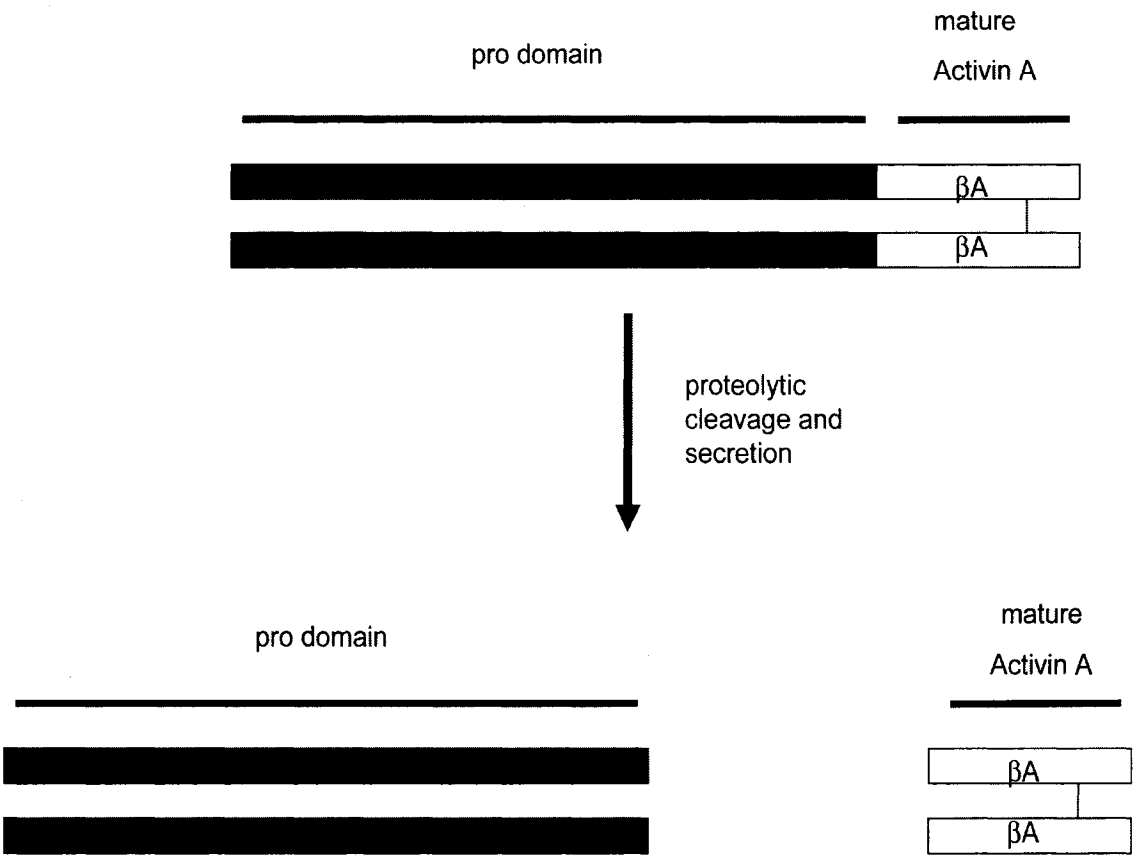


Figure 1-7. Proteolytic cleavage of mature Activin A. Mature Activin A is composed of two covalently bound βA subunits. Activins are synthesized as inactive precursor dimers containing pro domains and mature subunits. Proteolytic cleavage of the prodomains occurs inside the cell, where a mature and active Activin A dimer is secreted and can immediately induce signalling through interaction with Activin receptors.

CHAPTER 2 - MATERIALS AND METHODS

2.1 Cell Culture

2.1.1 Culture Of Primary Human OSE Or OvCa Cells

Institutional approval for research with human materials was received prior to the initiation of these studies (QEII Health Sciences Centre, Research Ethics Committee, #QE-RS-99-016; IWK/Grace Hospital Research Ethics). Primary human OSE cells were isolated and grown as previously described (Shepherd et al., 2006). Briefly, primary OSE cells were isolated from human ovary tissue samples through incubation in a Dispase II solution (Roche Canada) for 30 m with gentle agitation at 37°C. The tissue was then transferred in complete growth medium consisting of MCDB (Sigma Aldrich Canada) / Medium 199 (M199) (Invitrogen), supplemented with 5% fetal bovine serum (FBS) and 5% calf serum (Invitrogen), 100 mM L-glutamine (Invitrogen) and 1 X penicillin/streptomycin (P/S) (Invitrogen), and a scalpel blade was used to gently scrape the ovarian surface, releasing the cells from the top layer into the culture medium. Primary human OvCa cells were isolated from ascites fluid obtained from patients with stage III or IV OvCa, and grown as previously described (Shepherd et al., 2006) in MCDB/M199 medium supplemented with 5% FBS, 5% calf serum and 100 mM L-glutamine. All experiments with OvCa cells were performed between passages 2 to 6. A table summarizing the patient samples used in this study, as well as their associated diagnoses and type of analyses performed is located in Appendix 1.

2.1.2 Culture Of Primary Human Mesothelial Cells

MESO9 cells (a kind gift from Dr. C. Rancourt, Université de Sherbrooke), were cultured in DMEM/F12 medium (Invitrogen) supplemented with 10% FBS, 1 X P/S, 100

mM L-glutamine (Invitrogen), 400 ng/mL hydrocortisone (Sigma), and 10 ng/mL EGF (Sigma).

2.1.3 Culture Of The Human Fibroblast Cell Line F8

The human fibroblast cell line F8 was used in immunocytochemical analyses for the expression of cellular markers. These fibroblasts were maintained in DMEM (Invitrogen) supplemented with 5 % FBS and 100 mM L-glutamine.

2.2 Adenoviral Constructs And Cell Transduction

The constitutively active BMP Type IA receptor cDNA (ALK3QD; kind gift from Dr. L. Attisano, University of Toronto) was used to generate adenovirus expressing ALK3QD (Ad-ALK3QD) using the AdEasy Vector System (Qbiogene) according to the manufacturer's protocol. Ad-ALK3QD particles were purified using the BD Ad-X Virus Purification kit (BD Biosciences). Ad-GFP was a kind gift from Dr. B.C. Vanderhyden (Ottawa Health Research Institute). Primary OvCa cells were transduced with a multiplicity of infection (MOI) of 200 of Ad-ALK3QD or Ad-GFP in medium containing 10% FBS for 90 m with occasional agitation. After transduction, complete growth medium was replenished. ALK3QD is hemagglutinin (HA) epitope tagged; anti-HA was used to detect expression by western analysis (Figure 4-3).

2.3 Fluorescence And Confocal Microscopy

2.3.1 Immunocytochemistry And Microscopy For Vinculin And F-Actin

Primary OvCa cells were grown to 70-90% confluence on 18 mm diameter round glass coverslips placed in 22 mm diameter culture dishes. After treatment, growth medium was removed and cells were washed with phosphate-buffered saline (PBS), fixed

with 4% paraformaldehyde (PFA) in PBS for 10 m at room temperature (RT), and permeabilized with 0.3% Triton X-100 (Fisher) for 10 m at RT. Vinculin staining was done through indirect immunofluorescence employing a 1:100 dilution of a mouse anti-human vinculin primary antibody (Sigma) incubated with 10% donkey serum (Jackson Immunolabs) in PBS overnight at 4°C. Secondary antibody dilutions [1:1000 goat anti-mouse Alexa 488 (Molecular Probes)] were incubated with 0.05% Tween20 (Fisher), 0.1% BSA (Fisher) in PBS for 2 h at 37°C. Filamentous actin (F-actin) stress fibers were visualized by staining fixed and permeabilized OvCa cells with a 1:1000 dilution of either Alexa 488-conjugated Phalloidin or Rhodamine-conjugated Phalloidin (Molecular Probes) in 0.05% Tween20, 0.1% BSA for 1 h at 37°C, followed by incubation with Hoechst 33258 stain (1:1000 in PBS) for 15 m at 4°C to identify nuclei. Glass coverslips were permanently mounted onto glass slides using Biomeda Gel Mount (Fisher). Fluorescence images were obtained using a Zeiss Axiovert 200 inverted microscope with Zeiss Axiocam HRc digital camera at RT; 20 x objective has a numerical aperture of 0.5, 40 x objective has a numerical aperture of 0.6. Confocal images were taken using a Zeiss LSM 510 microscope equipped with Argon (458/488 nm) and Helium (548/633 nm) lasers, capturing 0.1 μm sections throughout the cell at RT; 63x objective has a numerical aperture of 1.4. Digital images were processed using Adobe Photoshop 7.0.

2.3.2 Indirect Immunocytochemistry For αSMA, Cytokeratins 7, 8, 18 And Vimentin

Cell culture, fixation, and permeabilization were performed as per section 2.3.1. A blocking solution of 10% donkey serum in PBS was then applied to the permeabilized cells for 1 h at RT with gentle agitation. Cells were then incubated with a primary antibody [1:1000 for mouse anti-human αSMA (Sigma), 1:500 for mouse anti-human

cytokeratins 7, 8 and 18 (Chemicon), and 1:1000 for mouse anti-human vimentin (Roche), as well a mouse IgG antibody at a 1:500 dilution to control for non-specific primary antibody interactions (Serotec) in an antibody dilution buffer consisting of 0.1% BSA, 0.05% Tween20 in PBS for 1 h at RT with gentle agitation. A goat anti-mouse Alexa 488-conjuagted secondary antibody (Molecular Probes) was then applied to the cells at a dilution of 1:2000 in antibody dilution buffer (0.1% BSA, 0.05% Tween20 in PBS) for 1 h at RT with gentle agitation, followed by incubation with the Hoechst 33258 stain (1:1000 in PBS) for 15 m at 4°C to identify nuclei. Glass coverslips were mounted and fluorescence images were obtained as in section 2.3.1.

2.4 RNA Isolation And QPCR

Total cytoplasmic RNA was isolated from cultured OSE and OvCa cells using the Sigma GenElute total mammalian RNA isolation kit (Sigma). cDNA was generated from 2 μg of total RNA by incubating with 0.5 μg oligo dT_{12-18} (Amersham), 10mM dNTPs, 0.1M DTT, and either 200 U of Superscript II reverse transcriptase (Invitrogen) or 50 U of Stratascript reverse transcriptase (Stratagene). Quantitative reverse-transcriptase polymerase chain reaction (QPCR) was performed using the Brilliant SYBR green® QPCR Master Mix and the Mx3000P Quantitative PCR machine and analysis software (Stratagene). Expression of mRNAs were assessed by QPCR (35 cycles: 30 s at 95°C, 30 s at 59°C, 30 s at 72°C) using primers specific for each human cDNA sequence tested (Table 2-1). The expression level for each cDNA was then normalized to its respective GAPDH mRNA expression level. Fold difference in mRNA expression before and after treatment was calculated using the normalized threshold of fluorescence (C_t) value obtained from the Mx3000P analysis software as previously reported (Livak and

Schmittgen, 2001). Data was obtained from a minimum of three independent experiments conducted in duplicate for each patient sample.

2.5 Wounding Assay

Normal OSE or primary OvCa cells were grown to 100% confluence on 18 mm diameter culture dishes or 25 mm square glass coverslips photo-etched with a lettered and numbered grid (Bellco Glass) in 35 mm diameter culture dishes, respectively. After treatment, growth medium was removed, cells were washed with PBS, and a wound was produced (~1 mm) by using the end of a plastic 10 μL pipette tip (Etienne-Manneville and Hall, 2001). Cells were rinsed with PBS to remove non-adherent cells, and fresh growth media with or without treatment was added. Digital images were taken at the same grid coordinates at time points up to 12 h with a Nikon TMS phase-contrast microscope equipped with a Nikon Coolpix 4500 digital camera. The percentage of the total area covered by the cells in each image was calculated using the NIH image analysis software program Image J. Assessment of motility was performed by subtracting the area at t=0h from the area at t=12h.

2.6 Adhesion Assay

Primary OvCa cells were cultured to 70-80% confluence and radiolabeled with a tritiated amino acid mixture (Amersham) in amino-acid depleted and low serum [0.1% newborn calf serum (Invitrogen)] growth medium (Roswell park memorial institute medium; RPMI; Sigma)] for 3 h. Cells were removed by brief trypsinisation, centrifuged, resuspended in complete growth medium (MCDB/M199) supplemented with 10% FBS, at a concentration of 100,000 cells per mL and allowed to recover in

suspension for 1 h at 37°C. Cells were plated at a density of 100,000 cells per well (24 well plate); wells were previously coated with 500 ng/cm^2 of ECM proteins [collagen I, collagen IV, fibronectin, laminin, vitronectin (Sigma)], allowed to air-dry and blocked with 1% BSA in PBS (vehicle) for 1 h at 37°C. Labelled cells were allowed to adhere at 37°C for up to 4 h, after which non-adherent cells and adherent cells were solubilized with a 0.1N NaOH solution. Acidified scintillation fluid was added to the cell samples, and disintegrations per minute (DPM) were counted using a Beckman/Coulter scintillation counter. Total cell number was calculated based on DPM measurements from a standard curve of radiolabelled cells.

2.7 Invasion Assay

Primary OvCa cells were cultured in 25 cm^2 flasks and pretreated with BMP4 (10 ng/mL) or transduced for 48h with either Ad-ALK3QD or Ad-GFP. Invasion assays were performed overnight (O/N) as previously described (Tan et al., 2004), where a collagen I (BD Biosciences) gel was pre-coated onto Transwell culture inserts. OvCa cells that had invaded through the Transwell culture insert filter were counted by fixing the cells with ethanol and staining with Mayer's hematoxylin (Sigma). Results are expressed as the mean number of cells/six high power fields of view that had invaded through the collagen gel and onto the culture insert filter.

2.8 Replating And Proliferation Assay

OvCa cells were treated with Vehicle (0.1% BSA in PBS) or BMP4 (10 ng/mL) for 48 h, trypsinized and replated in the presence or absence of BMP4. Cell morphology was visualized by phase contrast microscopy. Magnification 200x. OvCa cell

proliferation was determined by counting cell number using a haemacytometer throughout the growth period.

2.9 Rho-GTPase Activation Assays

Activation of Rac1, Rho, and Cdc42 GTPases were assayed using the Pierce guanosine triphosphate (GTPase) activation kits as previously described (Benard and Bokoch, 2002). Briefly, primary OvCa cells were grown to 70-80% confluence, incubated in low-serum conditions (0.2% FBS in growth medium) O/N, and treated for up to 48 h with 10 ng/mL BMP4 (R&D Systems). After treatment in low-serum conditions, GTPase activation was assessed as per manufacturer's instructions. Samples were run on a 15% polyacrylamide gel and transferred onto nitrocellulose membrane (Millipore). Rac1 (1:1000), Rho (1:500; detects Rho A, B, and C) and Cdc42 (1:500) were detected with monoclonal antibodies (Pierce kit). A 1:5000 dilution of sheep anti-mouse secondary antibody conjugated to horseradish peroxidase (HRP; Chemicon) was used to detect GTPase expression using enhanced chemiluminescence (ECL Plus) and STORM scanner digital imagery (Amersham-GE Healthcare).

2.10 Protein Extraction

Total cell protein extracts were isolated from primary OvCa cells grown in complete growth medium supplemented with 5% FBS and 5% calf serum (Invitrogen). After treatments, cells were lysed in buffer containing 50 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES) pH 7.4, 150 mM sodium chloride (NaCl), 10 % Glycerol, 1.5 mM magnesium chloride ($MgCl_2$), 1 mM ethyleneglycoltetraacetic acid (EGTA), supplemented with 1 mM Sodium orthovanadate, 10 mM sodium

pyrophosphate, 10 mM sodium fluoride (NaF), 1 % Triton X-100, 1% sodium deoxycholate, 0.1 % SDS, 1mM phenylmethanesulphonylfluoride (PMSF), and 1 X protease inhibitor cocktail (Roche - containing serine, cysteine and metalloproteases) at time of isolation. Cell lysis was performed at 4°C with gentle agitation for 10 m, followed by lysate collection and centrifugation at 13000 rpm for 20 m to clarify lysates and protein quantification using the Bradford protein assay (Bio-Rad).

2.11 Western Analysis

2.11.1 Phospho-Cofilin And Cofilin

Phospho-cofilin (p cofilin) and total cofilin (cofilin) protein expression in response to BMP4 treatment were assessed by electrophoresing 50 μg of total protein lysates on 15% sodium dodecyl sulfate (SDS)-polyacrylamice gels, transferred onto nitrocellulose membranes and blocked for 2 h at RT using 5% milk in Tris-buffered saline with Tween20 (TBST). Protein expression was detected using primary rabbit antibodies against human p cofilin (SER3) and cofilin (1:1000, Cell Signalling Technologies) in a solution of 5% milk in TBST at 4°C O/N with constant agitation. Both antibodies recognize a 19 kDa protein on western blots. A 1:5000 dilution of sheep anti-rabbit antibody conjugated to HRP (Chemicon) in 5% milk/TBST solution was then incubated with the blots for 2 h at RT with constant agitation. Specific protein expression was then detected using ECL Plus along with STORM scanner digital imagery (Amersham GE Healthcare).

2.11.2 Snail, Slug And E-Cadherin

Snail, Slug and E-cadherin protein expression in response to BMP4 treatment were assessed by electrophoresing 50 μg of total protein lysates on 15% (Snail and Slug) or 8% (E-cadherin) SDS-polyacrylamide gels, transferred onto nitrocellulose membranes and blocked for 2 h at RT using 5% milk in TBST. Protein expression was detected using primary rabbit antibodies against human Snail, Slug (1:100; Santa Cruz Biotechnologies Inc.), and E-cadherin (1:500; Cell Signalling Technologies) in a solution of 5% BSA in TBST at 4°C O/N with constant agitation. Snail and Slug antibodies recognize a 29 and 30 kDa protein respectively, while E-cadherin antibodies recognizes a 135 kDa protein on western blots. A 1: 5000 dilution of sheep anti-rabbit antibody conjugated to HRP (Chemicon) in 5% BSA/TBST solution was then incubated with the blots for 2 h at RT with constant agitation. Specific protein expression was then detected using ECL Plus along with STORM scanner digital imagery (Amersham GE Healthcare).

2.11.3 Smads Proteins And Id1

Total protein was isolated as per section 2.11 and 40 μg of total OvCa cell lysates were electrophoresed on 10% polyacrylamide gels and transferred onto nitrocellulose membrane. After blocking the membranes with 5% milk/TBST for 2 h at RT, rabbit anti-human phospho-Smad1 (SER 463/465) / Smad5 (SER 463/465) / Smad8 (SER 426/428) (1:1000; Chemicon), rabbit anti-human phospho-Smad 2 (1:1000; Cell Signalling Technologies), rabbit anti-human Smad1, rabbit anti-human Smad 2, mouse anti-human Smad 5 (1:250; Zymed laboratories), goat anti-human Smad 8, and rabbit anti-human Id1 (1:100; Santa Cruz Biotechnologies) were incubated in 5% BSA/TBST solution O/N at 4°C with constant agitation. The total Smad 1, 5 and 8 antibodies

recognize a 52 kDa protein, the total Smad 2 antibody recognizes a 56 kDa protein, the p Smad 2 antibody recognizes a 60 kDa protein, and the p Smad 1/5/8 antibody recognizes a 65 kDa protein on western blots. Blots were then incubated with either a 1:5000 dilution of sheep anti-rabbit-HRP antibody or goat anti-mouse-HRP antibody in 5% BSA/TBST for 2 h at RT with constant agitation, followed by ECL Plus detection and STORM scanner digital imagery.

2.11.4 GAPDH

With all of the western blot protein determinations, protein loading was verified by incubating blots with a mouse anti-human glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:10000; Chemicon) antibody in a solution of 5% milk/TBST O/N at 4°C with constant agitation. The GAPDH antibody recognizes a 36 kDa protein on western blots. Blots were then incubated with a 1:5000 dilution of goat anti-mouse-HRP antibody in 5% milk/TBST for 2 h at RT with constant agitation, followed by ECL Plus detection and STORM scanner digital imagery. The GAPDH protein determinations were performed immediately after any of the other protein determinations without stripping the membrane, as none of the other proteins assayed shared the same molecular weight.

2.12 Growth Curves

Primary OvCa cells were seeded at low density (2 x 10^4 cells/mL) onto triplicate 6-well culture dishes. The next day (Day 0) cells were counted using a Coulter counter (Beckman Coulter Canada) and cells were treated in complete growth medium on Day 0. Culture medium and treatments were replenished every 2 days, and cell counts were measured every 2 days subsequent to treatment until 6 days post-treatment. Cell counts for each day and treatment were obtained by averaging cell counts from triplicate wells.

Digital cell images at day 6 post-treatment were taken using a Nikon TMS phase-contrast microscope equipped with a Nikon Coolpix 4500 digital camera.

2.13 Lentivirus Constructs And Cell Transductions

Small hairpin RNA (shRNA) molecules against Smads 1, 4, and 5 were designed and obtained through Open Biosystems (The RNAi Consortium) already contained within their expression vector hairpin-pLKO.1 (Smad 1 – TRCN0000021783, Smad 5 – TRCN0000057049, Smad 4#1 – TRCN0000010321, Smad4#2 – TRCN0000040032). Additionally, a scramble shRNA pLKO.1 construct was obtained (Addgene ID#1864) to use as a negative control, as this scramble construct contains a shRNA molecule that does not target a particular mRNA sequence. Sequences of the shRNA molecules that were used are listed in Table 2-2. Each of the hairpin-pLKO.1 constructs was co-transfected with a packaging plasmid (psPAX2, Addgene ID#12260) and an envelope plasmid (pMD2.G, Addgene ID#12259) in order to produce viable lentiviral particles in HEK-293T cells. The purified media obtained from HEK-293T transduction containing virus particles was then used to transduce cultures of primary human OvCa cells. For protein and morphology determinations, OvCa patient cell samples were seeded at a density of 2 x 10^4 onto 60 mm dishes, and treated the following day with 250 µL or 500 µL of viral suspension, when the cells were 40-50% confluent. The viral particles were left on the cells O/N (approximately for 18 h), and a full media change was completed the next morning. After 48 h of culture, the cells were then treated with vehicle or BMP4 at 10 ng/mL. Total protein was isolated after 90 m of treatment, while morphology was assessed by culturing the cells for a total of 4 d in the presence or absence of BMP4 and by taking digital pictures every day. Media was replenished every 2 days with fresh

vehicle or BMP4. For motility determinations, OvCa patient cell samples were seeded in 6-well dishes, and transduced with 200 μL/well of lentiviral suspension for approximately 18 h, after which a complete media change was performed. After 48 h of culture, cells were lifted and re-plated at a lower density (3 x 10^4 cells/mL) into 6-well dishes containing etched-grid coverslips. The next day, cells were pre-treated with either vehicle or BMP4 at 10 ng/mL for 48 h. After the treatment period, a wounding assay was performed as described in section 2.5.

2.14 Statistical Analyses

For all experiments where *P* values are expressed, a paired Student's t test was performed, where significance was set at $P < 0.05$. Unless otherwise stated, the *P* values represent data obtained from 3 independent experiments performed in triplicate.

Target	Forward primer (5')	Reverse primer (3')	product size (bp)	NCBI accession #
α4 integrin	CCCCAGGATCATCTTACTGGA	TATGCTGGCTCCGAAAATGAC	150	NM_000885
α2 integrin	GCAACTGGTTACTGGTTGGTT	GAGGCTCATGTTGGTTTTCATCT	167	NM_002203
β1 integrin	GGGACACGCAAGAAAATCCGA	GCTTTACGTCCGTAGTTTGGA	73	NM_033668
αV integrin	CATCTGTGAGGTCGAAACAGG	TGGAGCATACTCAACAGTCTTTG	137	NM_002210
α1 integrin	GAAAACGTGACCCATGAGTTCA	TCCTTTCTTGCTGTGTCTATTCC	134	NM_181501
α5 integrin	GCCTGTGGAGTACAAGTCCTT	AATTCGGGTGAAGTTATCTGTGG	163	NM_002205
α3 integrin	TCTCTCCTTGGCCACAGACT	TCCCAACATCTGTCTGTCCA	153	NM_005501
β3 integrin	GCTATGGTTCTCTCGCAAGG	AGAGCTGCCAATAAGGCAAA	161	NM_000212
Collagen I	ATGGATTCCAGTTCGAGTATGGC	CATCGACAGTGACGCTGTAGG	246	NM_000088
Collagen IV	AGAGATTGCTCTGTTTGCCAC	CGGTCCCCTCTCATTCCTT	143	NM_000092
Laminin	CAGTGACCAGATTCCCCTGT	ACATTCGCATCAACCACAAA	235	AF005258
Fibronectin	CAGTGGGAGACCTCGAGAAG	TCCCTCGGAACATCAGAAAC	168	NM_002026
Vitronectin	GCGGGGATGTGTTCACTATG	GTGTCTGCTCAGGATTCCCTT	150	NM_000638
Vinculin	ACACTCTGCTCTGCCCTTGT	CAGCAACATGGAAAGCTCAA	166	NM_014000
Talin	TGGGCTCTTTCTGTCAGATGA	CCATGATCGTCTTCACAGTTCC	164	NM_006289
FAK	GGACCAGGAAATTGCTTTGA	TTTTGGCCTTGACAGAATCC	167	L13616
Filamin	TGTACCGCAAGTACCATCAGC	ACCAGACCCAAGATGAGCTTC	157	NM_001457
Snail	AATCGGAAGCCTAACTACAGCG	GTCCCAGATGAGCATTGGCA	147	NM_005985
Slug	AAGCATTTCAACGCCTCCAAA	AGGATCTCTGGTTGTGGTATGAC	119	NM_003068
E-cadherin	CCCACCACGTACAAGGGTC	CTGGGGTATTGGGGGCATC	94	NM_004360
Alk1	TTGAGGAAATGCGAAAGGTTGT	GCTGGGAGAGGGTCTTCTTGA	171	NM_004302
Alk2	AAAGGCTGCTTCCAGGTTTATG	CCGTGATGTTCCTGTTACACC	112	NM_001105
Alk3	CACTGCCCCCTGTTGTCATAG	ATCCTGTTCCAAATCACGATTGT	179	NM_004329
Alk4	AAAGGCCGCTATGGCGAAG	CGTGTCTGAGCAATACTGTGT	133	NM_000020
Alk5	TGTTGGTACCCAAGGAAAGC	CACTCTGTGGTTTGGAGCAA	160	L11695
Alk7	GTGGCTGGAATGGTCAAGTT	TTTCACAGCCAGCCCTAAGT	177	U69702
ActR-IIA	TTGCTGTGAGGGCAATATGTG	GTTGTAATAGGGTGGCTTAGGTG	106	NM_001616
ActR-IIB	TTCAACTGCTACGATAGGCAGG	GCCTCTGGCAAATGAGTGAAG	113	NM_001106
TGFbetaRI	AGTGTGCTTCGTCTGCATCTC	GTCTTTCAACGTAGTACCCTCTG	139	NM_004612
Noggin	ATCGAACACCCAGACCCTATC	TCTAGCCCTTTGATCTCGCTC	233	NM_005450
BAMBI	AGCACGACAGACATCTGCC	AGCATTCCAATGTGGGTATGG	73	NM_012342
Chordin	GGGCTGCTATTTTGATGGTG	CCCCGAGCCACAGGACAGT	88	NM_177978
Follistatin	ACGTGTGAGAACGTGGACTG	CACATTCATTGCGGTAGGTTTTC	151	NM_006350
Gremlin	AAGGGAAAAAGAAAGGGTCCCA	GTCTGCTCTGAGTCATTGTGC	73	NM_013372
ID1	CTGTCTGAGCAGAGCGTGG	TAGTCGATGACGTGCTGGAG	200	NM_002165
ID3	TGGTTTTCTTTCTCTTTGGGG	CGGGAGTAGCAGTGGTTCAT	193	NM_002167
CUTL-1	TACCCCTGGCTTCTACAGGAA	GGCTTTGTTGTAATGGACTTTGG	171	NM_181552
GAPDH	CATGAGAAGTATGACAACAGCCT	AGTCCTTCCACGATACCAAAGT	113	J04038

Table 2-1. Sequences of primers used in QPCR.

shRNA	hairpin sequence (5' to 3')	target mRNA (NCBI accession #)
Smad4 #1	CCGGTACCATACAGAGAACATTGGACTCGAGTCCAATGTTCTCTGTATGGTATTTTTG	NM_005359
Smad4 #2	CCGGGTACTTCATACCATGCCGATTCTCGAGAATCGGCATGGTATGAAGTACTTTTTG	NM_005359
Smad 1	CCGGCCAGCTGTGAAGAGACTTCTTCTCGAGAAGAAGTCTCTTCACAGCTGGTTTTT	NM_005900
Smad 5	CCGGGCCGAGTAAATGTGTCACTATCTCGAGATAGTGACACATTTACTCGGCTTTTTG	NM_001001419
Scrambled	CCTAAGGTTAAGTCGCCCTCGCTCTAGCGAGGGCGACTTAACCTTAGG	none

Table 2-2. Sequences of shRNA molecules used in lentivirus transductions. Sequences of hairpin shRNAs are listed; red represents the 'sense' sequence, green represents the loop sequence, and blue represents the 'antisense' sequence.

CHAPTER 3 - VERIFYING THE ORIGIN OF CELLS TO STUDY OVARIAN CANCER *IN VITRO*

3.1 SUMMARY

An immunocytochemical staining and real-time quantitative PCR procedure was developed to differentiate between primary human cultures of normal OSE and OvCa cells. This procedure identifies a differential expression pattern of cytokeratins 7, 8 and 18, vimentin, alpha smooth muscle actin (αSMA), and the mRNA of the cut-like 1 (CUTL-1) transcription factor between primary normal OSE and OvCa cells. Immunocytochemical analyses revealed that normal OSE cells express cytokeratins 8 and 18, while OvCa cells express cytokeratins 7, 18 and vimentin. This study was extended to assess the expression profile of normal mesothelial cells and fibroblasts and revealed that mesothelial cells express cytokeratin 18, vimentin and αSMA, while fibroblasts did not express any of the cytokeratins but did express vimentin and αSMA. The mRNA expression level of CUTL-1 was increased 4-fold in OvCa cells as compared to normal OSE, while CUTL-1 expression in fibroblasts was decreased 20- and 6-fold as compared to OvCa cells and normal OSE cells, respectively. Taken together, these results indicate that the expression profile of 6 markers can discriminate between normal OSE and OvCa cell cultures, as well as identify potentially contaminating cells that may be present following sample isolation and culture. This procedure is sufficient to confirm the identity and purity of primary cell cultures from normal and cancerous patients for comparative analyses.

3.2 INTRODUCTION

The use of cellular protein and molecular expression profiles to differentiate cancerous from normal cells has proved useful in the clinic to diagnose a variety of epithelial malignancies (Barak et al., 2004). Our laboratory employs the culture of primary human OvCa cells from the ascites of patients, and primary normal OSE cells isolated from normal ovaries as a model system to study signalling pathways that may be affecting OvCa cell biology (Shepherd et al., 2006). It is thought that most OvCas arise from the OSE (Auersperg et al., 2001), and as such, we conduct comparative analyses of the responses between normal OSE and OvCa cells to identify crucial differences that may help us determine the mechanisms of OvCa etiology and progression. The origin of the cells that we obtain, in addition to the methods we use to isolate and culture these cells *in vitro*, allow for minimal contamination of our cultures with non-epithelial cell types (Shepherd et al., 2006). In order to fully characterize our OvCa and normal OSE cell cultures obtained from patients, we have developed a simple, yet effective method to confirm the identity of our cell cultures for comparative analyses.

Cytokeratins are a subfamily of intermediate filament (IF) proteins that are mainly expressed in cells of epithelial origin. Cytokeratin expression will vary with epithelial cell type and differentiation state, and may change during cancer progression, allowing for the identification of tumor cells within normal tissues (Moll, 1994). Vimentin is also an IF that is co-expressed along with other cytokeratins in ovarian carcinoma tissue samples (Dabbs and Geisinger, 1988). It is also expressed by cells of mesenchymal origin, such as mesothelial cells and fibroblasts (Czernobilsky et al., 1985). Mesothelial cells compose the peritoneal wall lining, and these cells may be a potential source of

contamination in the ascites fluid that we obtain from OvCa patients. Additionally, fibroblasts reside within the stroma of many organs contained within the peritoneal cavity including the ovary, and may be a source of contaminating cells in our cultures from ascites fluid and normal ovarian tissue. The expression profile of cytokeratins and vimentin has been used in many studies to evaluate cell types in normal ovary and OvCa tissue samples (Auersperg et al., 2001; Czernobilsky et al., 1985; Dabbs and Geisinger, 1988); however in cell culture, very few studies have assessed the expression of these proteins from primary normal OSE and OvCa cells obtained from patient samples (Jiang et al., 2003; Ramaekers et al., 1990).

This chapter defines an immunocytochemical staining and quantitative real-time PCR (QPCR) procedure identifying a differential expression of cytokeratins (7, 8, and 18), vimentin, αSMA and the mRNA of the transcription factor CUTL-1 between primary human normal OSE and primary human OvCa cells. We also show a differential marker expression profile when OSE and OvCa cell expression profiles are compared to those of primary human mesothelial cells (MESO9) and the human F8 fibroblast cell line. This panel of markers is what we routinely use to consistently identify the cellular composition of our primary cultures. The differential expression pattern exhibited by OvCa and normal OSE patient samples allows us to identify cultures that meet our criteria for subsequent analyses, and effectively confirm the cellular purity of our primary cultures.

3.3 MATERIALS AND METHODS

Isolation and culture of primary human normal OSE and OvCa cells were performed as outlined in section 2.1.1. Culture of primary human mesothelial cells (MESO 9) was performed according to section 2.1.2; the culture of the human fibroblast cell line F8 was performed according to section 2.1.3. Immunocytochemical staining of cytokeratins 7, 8 and 18, as well as αSMA and vimentin were performed according to section 2.3.2. This analysis consisted of assessing the immunocytochemical expression profile of one OSE sample (OSE # 108), 4 OvCa samples (OvCa # 85, 89, 92, and 103), one mesothelial sample (MESO 9) and one human dermal fibroblast sample (F8). Only one OSE sample was used in the immunocytochemical analyses due to the limited availability of these primary patient samples. Assessment of marker expression through fluorescence microscopy was determined on a total of 3 slides per protein marker, on each of the patient samples and cell lines tested. A minimum of 20 cells/slide were observed for marker expression. In each slide, over 95% of the cells that were observed showed the same expression pattern for each marker tested. Less than 5% of cells visualized exhibited lower or no expression of the marker in question. Cytokeratin expression was considered non-existent when the fluorescence intensity exhibited by the various cell markers was equivalent to the intensity of fluorescence staining for the IgG control. RNA isolation and mRNA expression of CUTL-1 was performed as outlined in section 2.4 with 12 normal OSE and 10 OvCa primary patient samples, in addition to one OvCa cell line (CaOV3), MESO9 and F8 dermal fibroblasts. In order to evaluate CUTL-1 mRNA expression for each individual cell sample tested, mRNA levels were

normalized to their respective GAPDH mRNA expression, and plotted as relative expression units. Statistical analyses were conducted as in section 2.14.

3.4 RESULTS

3.4.1 Normal OSE Cell Expression Profile

Immunocytochemical analysis of normal OSE marker expression revealed that over 95% of the OSE cells observed express cytokeratins 8 and 18 (Figure 3-1). These results are in accordance with the expression profile of normal epithelial cells, as cytokeratin 7 is mainly expressed on carcinoma cells (Cathro and Stoler, 2002), while cytokeratin 8 is expressed on cells of simple epithelia (Moll et al., 1982). Cytokeratin 18 is typically expressed on simple epithelia, but can also be expressed on cells of mesenchymal origin or adenocarcinomas (Moll et al., 1982) and its expression has been shown on normal OSE cells and OvCa cells (Auersperg et al., 2001). Normal OSE cells showed no expression of vimentin or αSMA, indicating that our OSE cultures are most likely of normal epithelial cell origin.

3.4.2 OvCa Cell Expression Profile

Primary human OvCa cells obtained from 4 different patient samples show a similar expression profile, where over 95% OvCa cells assessed express cytokeratin 7, 18 and vimentin. These cells showed no cytokeratin 8 or αSMA expression (Figure 3-1), indicating that our OvCa cell cultures are carcinoma cells.

3.4.3 Mesothelial And Fibroblast Cell Expression Profiles

To compare and contrast the expression profile of our normal OSE cell and OvCa cells to those of cells that are of a different source and type, we evaluated the expression of proteins identified by the immunocytochemical panel on a primary normal mesothelial cell culture (MESO 9), and a normal human dermal fibroblast cell line (F8). Most (over 95%) MESO 9 cells expressed cytokeratin 18, vimentin and αSMA, while over 95% of

F8 fibroblasts did not express any of the cytokeratins but expressed both vimentin and αSMA (Figure 3-2). This expression profile confirms the nature of these two cell types; that MESO9 cells possess characteristics of both epithelial and mesenchymal cells, while F8 fibroblasts are cells of mesenchymal origin.

3.4.4 CUTL-1 mRNA Expression

The transcription factor CUTL-1 is overexpressed in many cancers, and has been shown to be a putative predictor of tumor aggressiveness in breast and pancreatic cancers (Michl et al., 2005; Neville et al., 2001). CUTL-1, also called CDP (CCAAT displacement protein), Cut, or Cux-1 is a homeodomain transcription factor that is a regulator of DNA binding activity. It has been identified as a transcriptional regulator of cellular motility and invasiveness in cancer cells (Michl et al., 2005). The locus of this gene maps to the 7q22 region, where studies have shown loss of heterozygosity (LOH) of this region in ovarian tumors (Neville et al., 2001). The expression of this molecule may therefore be a distinguishing feature between normal OSE and OvCa cells. To determine whether the expression of CUTL-1 was different between normal OSE, OvCa cells, mesothelial and fibroblasts, an analysis of CUTL-1 mRNA was performed using QPCR. A 4-fold increase in CUTL-1 mRNA expression was shown in OvCa patient samples as compared to normal OSE samples (Figure 3-3, $P < 0.05$). Furthermore, no difference was seen in mean CUTL-1 expression between primary OvCa cells and the OvCa cell line CaOV3. Although MESO9 CUTL-1 expression did not differ from OvCa cell expression, there was a significant decrease in CUTL-1 mRNA between F8 fibroblast as compared to OvCa cells (~95% decrease in CUTL-1 expression in F8 cells versus OvCa cells, and ~ 80% decrease as compared to normal OSE cells, $P < 0.05$). These results

indicate that CUTL-1 expression can be a differentiating factor between normal OSE, OvCa and fibroblast cells.

3.5 DISCUSSION

We have shown that an immunocytochemical expression pattern of 5 different molecules can differentiate cells of epithelial origin from those of mesenchymal origin. In this way, we are able to identify cells of non-epithelial origin that may be present in our samples obtained from patients when these are cultured, and discard these patient samples from future analyses. Furthermore, based on the differential cytokeratin expression panel between normal OSE and OvCa cell samples, we can clearly identify and confirm the nature of our cells once propagated in culture. We have also identified another potential molecular marker, CUTL-1, whose mRNA is highly expressed in our OvCa patient samples as compared to our normal OSE samples. Through this simple yet effective method of evaluating the cellular nature of our cultures, we are confident that we are employing the appropriate cell types for comparative analyses.

The differential expression of cytokeratins 7, 8, 18, vimentin and αSMA has allowed us to discriminate between normal OSE and OvCa cell cultures. Normal OSE cells express cytokeratins 8 and 18, which are present in simple epithelial cells. Normal OSE cultures express cytokeratin 8, however the OvCa cell cultures do not. This is an interesting result given the fact that cytokeratin 8 can also be expressed in both normal epithelium and carcinoma cells (Moll et al., 1982); this difference in cytokeratin expression pattern may simply reflect the culture conditions that our laboratory employs. Nevertheless, this result was consistently seen when 4 different OvCa patient samples and one normal OSE sample were assessed, showing that the expression of cytokeratin 8, in our hands, can be a distinguishing feature between normal OSE and OvCa cell cultures. Normal OSE cells also express cytokeratin 18, which has been shown to be

expressed on both epithelial and mesenchymal cell types (Mutsaers, 2004; Pittenger et al., 1999). Vimentin and αSMA are not present in normal OSE cells, further confirming the epithelial nature of these cell cultures.

Primary human OvCa cells express cytokeratin 7, which is present in OvCa tissues and has been used in the past to distinguish between normal and cancerous tissue in OvCa (Cathro and Stoler, 2002; Dabbs and Geisinger, 1988), as well as distinguish between ovarian carcinoma and other adenocarcinomas (Ramaekers et al., 1990). In addition to cytokeratin 7, our OvCa cells express cytokeratin 18 and vimentin. Cytokeratin 18 is a common marker of simple epithelial cells, and has been previously shown to be expressed on normal OSE and OvCa cells (Auersperg et al., 2001). Vimentin has been shown to be co-expressed with cytokeratins in OvCa tissues, making the expression of this molecule another distinguishing feature between normal OSE cells and OvCa cells (Dabbs and Geisinger, 1988). Primary OvCa cells do not express αSMA, confirming that these cells are likely not of mesenchymal origin.

In order to confirm the specificity of our antibody panel to detect cells of mesenchymal origin, we also assayed the expression of primary human mesothelial cells and a dermal fibroblast cell line. Mesothelial cells form the peritoneal lining surrounding the abdominal cavity, and they are an unusual cell type in that they maintain characteristics of both epithelial and mesenchymal cell types (Mutsaers, 2004). Our MESO9 cultures expressed cytokeratin 18, vimentin and αSMA. Cytokeratin 18 and vimentin can be expressed on both epithelial and mesenchymal cell types, while αSMA is a mesenchymal cell marker. Our results show that indeed, primary mesothelial cells in culture maintain both an epithelial and a mesenchymal cell phenotype that is different

from the expression panel shown by both normal OSE and OvCa cells in culture. Interestingly, fibroblasts assayed in our study did not express cytokeratin 18, but did express vimentin and αSMA, which are markers expressed on cells of mesenchymal origin. Fibroblasts form the stromal component of many tissues within the peritoneal cavity, including the ovary. These results confirm that our fibroblast cultures are of mesenchymal lineage, and that their differential staining pattern can distinguish these cells in culture.

The transcription factor CUTL-1 is differentially expressed in normal OSE, primary OvCa cells and cell lines, as well as in fibroblasts. This molecule has been shown to be overexpressed in many cancers, including breast and pancreatic (Michl et al., 2005; Neville et al., 2001), and is overexpressed in our OvCa cultures when compared to normal OSE cells. We have thus identified an OvCa cell marker that can distinguish between normal OSE and OvCa cells. Additionally, CUTL-1 expression is significantly lower in fibroblasts than in normal OSE or OvCa cells, identifying another distinguishing feature between these two cell types. CUTL-1 expression was not significantly different between OvCa cells and mesothelial cells; although the mesothelial cells were obtained from the peritoneal lavage of a patient with no known history of OvCa (Dr. C. Rancourt, personal communication), these data indicate that CUTL-1 mRNA expression may not be an appropriate molecular marker to solely discriminate between primary OvCa and mesothelial cells. Other protein marker expression profiles (such as with the differential expression of cytokeratins) would be required to effectively discern these two cell types. The expression of CUTL-1 can be determined through QPCR, requiring much less cellular material and time than with immunocytochemical analyses. Although CUTL-1

mRNA expression was shown to be variable within normal OSE and OvCa patient samples, the overall difference in the mRNA expression of CUTL-1 from pooled normal OSE and OvCa patient samples has proven to be a significant distinguishing feature between these two cell types, and thus can be employed as an additional cell marker in identifying the nature of the primary cultures. The increase in CUTL-1 expression exhibited by OvCa cells and cell lines as compared to normal OSE cells is most likely not a reflection of cell proliferation, as CUTL-1 expression is similar between these two cells types, but the growth rate of OvCa cell lines is much faster than primary OvCa cells (Shepherd et al., 2006). Taken together, these results confirm that our immunocytochemical staining and mRNA expression panel can recognize potentially contaminating cell types that could be present in preparations of both normal OSE and OvCa ascites samples. As previously mentioned, cytokeratin expression can vary with epithelial cell type and differentiation state, but also with cell culture conditions (Moll, 1994). However, the fact that the same cytokeratin profile was obtained with 4 different OvCa patient samples with varying diagnoses indicates that the culture conditions in which the cells are grown effectively maintain a consistent profile of cytokeratin expression, allowing for the differentiation between cells types that could exist within these primary cell isolations. Additionally, over 95% of the cells visualized in each cell sample that was tested exhibited the same expression profile, further confirming the purity of our cultures for comparative purposes. The use of this panel of 6 cell markers is sufficient for us to be confident of the identity and purity of our primary cell cultures obtained from normal and cancerous patients for use in comparative *in vitro* analyses.

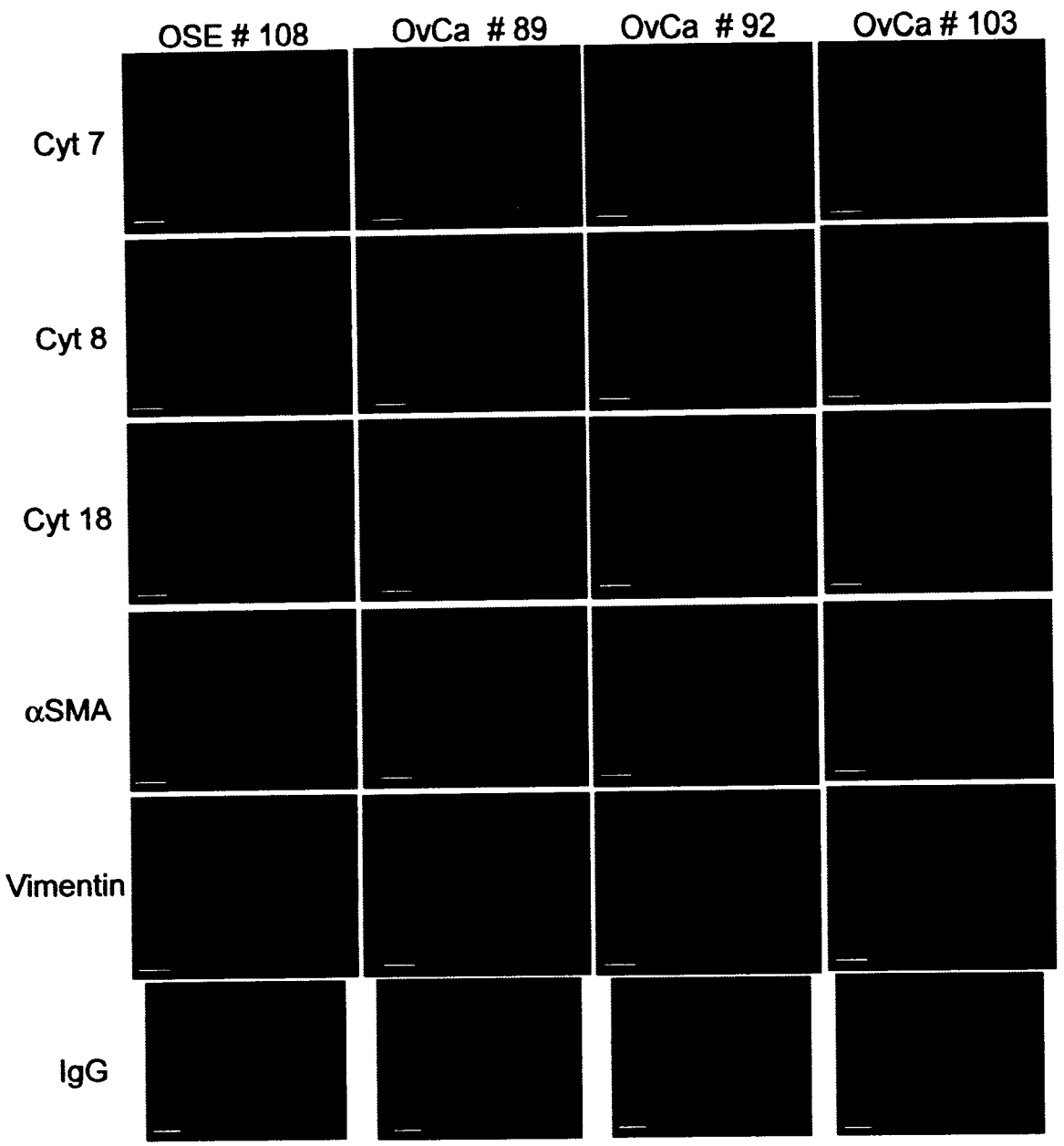


Figure 3-1: Cytokeratin, αSMA, and vimentin expression profiles of normal OSE and OvCa patient samples. OSE (#108) and 3 samples of OvCa cells (#89, #92, and #103) were stained with antibodies against cytokeratins 7 (Cyt 7), 8 (Cyt 8), 18 (Cyt 18), alpha smooth muscle actin (αSMA), vimentin and an IgG_1 control antibody (IgG) plus an Alexa 488-conjugated secondary antibody (green), visualized through fluorescence microscopy. Cells were co-stained with Hoechst 33258 to reveal nuclei (blue). A total of 4 different OvCa patient samples were assessed, all with similar results. Bar, 50 μm.

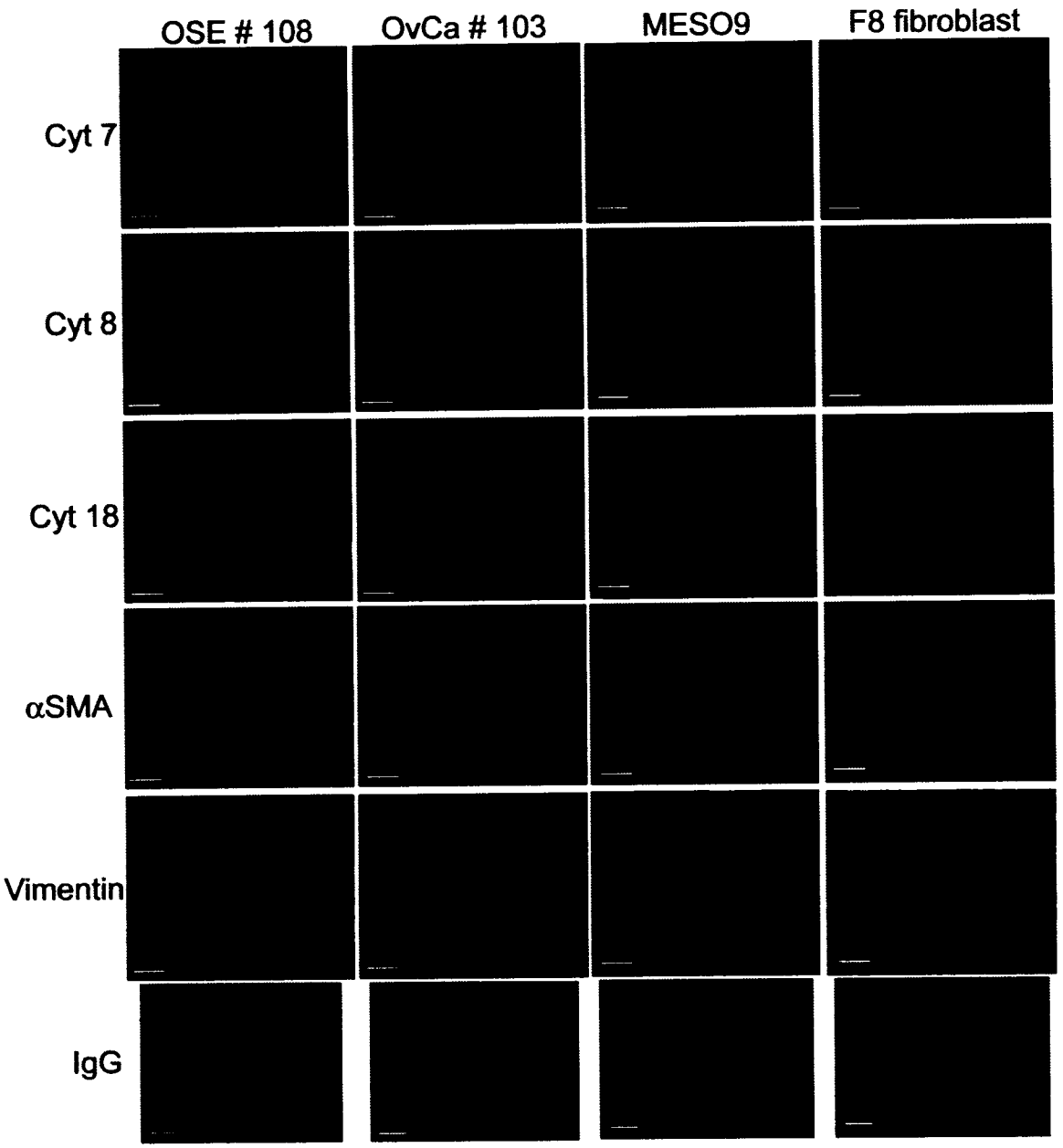
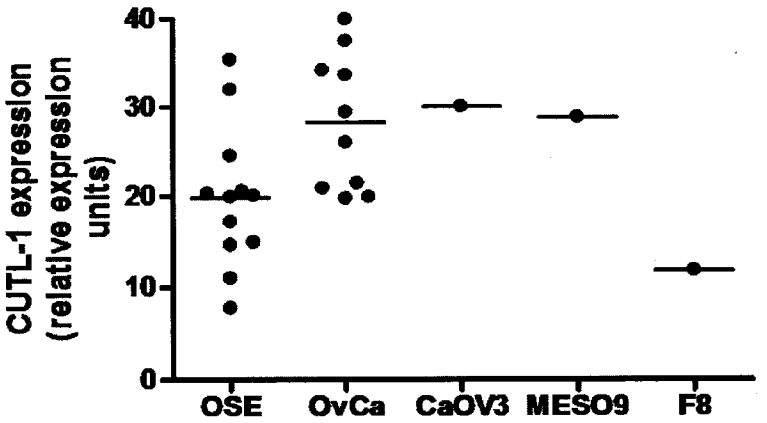


Figure 3-2: Cytokeratin, αSMA, and vimentin expression profiles of normal OSE, OvCa, mesothelial and fibroblast cells. OSE (#108), OvCa cells (#103), primary human mesothelial cells (MESO9) and a human fibroblast cell line (F8) were stained with antibodies against cytokeratins 7 (Cyt 7), 8 (Cyt 8), 18 (Cyt 18), alpha smooth muscle actin (αSMA), vimentin and an IgG_1 control antibody plus an Alexa 488-conjugated secondary antibody (green), visualized through fluorescence microscopy. Cells were co-stained with Hoechst 33258 to reveal nuclei (blue). A total of 4 different OvCa patient samples were assessed, with similar results. Bar, 50 μm.

A



B

Sample #	CUTL-1 expression
OSE #3	19.92
OSE #4	31.95
OSE # 5	20.30
OSE# 22	17.18
OSE #23	35.27
OSE # 32	20.53
OSE # 42	11.01
OSE # 43	20.08
OSE # 45	24.51
OSE # 49	14.68
OSE # 65	14.99
OSE # 89	7.79
OvCa # 28	37.45
OvCa # 45	29.41
OvCa # 46	39.92
OvCa # 64	19.92
OvCa # 69	19.76
OvCa # 70	20.88
OvCa # 71	33.56
OvCa # 87	34.13
OvCa # 92	21.44
OvCa # 103	26.01
CaOV3	30.03
MESO9	28.82
F8	11.90

Figure 3-3. CUTL-1 mRNA expression in normal OSE, OvCa, mesothelial and fibroblast cells. (A) 12 primary normal OSE cell samples, 10 primary OvCa cell samples, 1 OvCa cell line (CaOV3), 1 primary mesothelial (MESO9) cell sample and 1 fibroblast cell line (F8) were assessed for CUTL-1 expression using QPCR. Expression levels for each sample (normalized to its respective GAPDH level) are represented (black dots) along with the mean expression values (black line) for each cell type, expressed as relative expression units. **(B)** Table depicting CUTL-1 expression values for each sample tested in **(A)**.

CHAPTER 4 – BMP4 INDUCES EMT AND RHO GTPASE ACTIVATION IN HUMAN OVARIAN CANCER CELLS

[Portions of this chapter appeared in the following publication and have been reprinted with permission: Thériault *et al.* 2007. BMP4 induces EMT and Rho GTPase activation in human ovarian cancer cells. Carcinogenesis 28 (6): 1153-1162.]

N.B.: The co-authors Dr. Trevor G. Shepherd and Dr. Michelle L. Mujoomdar contributed to part of the work presented in this chapter. Dr. Shepherd provided the Ad-ALK3QD western blot depicting the MOI in primary OvCa cells in figure 4-3, XIII, and Dr. Mujoomdar assisted the primary author with the execution and analysis of the invasion experiments depicted in figure 4-15.

4.1 SUMMARY

We previously identified an autocrine BMP4 signalling pathway in primary human normal ovarian OSE and OvCa cells. Herein we show that treatment of OvCa cells with BMP4 produced morphological alterations and increased cellular adhesion, motility, and invasion. The BMP4 inhibitor Noggin blocked the BMP4-induced phenotype, and decreased autocrine BMP4 mediated OvCa cell motility and adherence. In response to exogenous BMP4, the EMT markers Snail and Slug mRNA and protein were upregulated, E-cadherin mRNA and protein was downregulated, and the network of αSMA changed to resemble a mesenchymal cell. We also observed changes in the level of activated Rho-GTPases in OvCa cells treated with BMP4, strongly suggesting that the changes in morphology, adhesion, motility and invasion are likely mediated through the activation of these molecules. Strikingly, treatment of normal OSE cells with BMP4 or Noggin failed to alter cell motility, providing evidence that OSE and OvCa cells possess a distinct capability to respond to BMP4. Furthermore, we show that the BMP4-induced changes in morphology and motility are Smad-dependent through the inhibitory RNA-mediated targeting of Smad expression. Overall, our studies suggest a link between autocrine BMP signalling mediated through Smad-dependent signalling, the Rho-GTPase family, and Snail and Slug-induced EMT that may collectively contribute to aggressive OvCa behaviour.

4.2 INTRODUCTION

BMPs are expressed in many adult tissues and play important functions in adult tissue formation, maintenance, remodeling, and repair (Balemans and Van Hul, 2002; Chen et al., 2004; Hogan, 1996). BMP dimers transduce their signals through a heterotetrameric complex consisting of type I (ALK2, 3, 6) and type II (BMPRII) serine/threonine kinase transmembrane receptors. Subsequent intracellular signalling is mediated through activation of Smads 1, 5 and 8 interacting with the common Smad 4, and upon nuclear translocation modifies target gene expression (Attisano and Wrana, 2002). Many BMPs are expressed in the ovary (BMP 2, 3, 3b, 4, 6, 7, 15) and contribute to the processes of follicle maturation and steroidogenesis (Knight and Glister, 2003; Shimasaki et al., 2004). BMP4 in particular is expressed in a spatially and temporally distinct manner in the OSE layer of cells immediately following ovulation, and based on this expression profile it was suggested that BMP4 signalling may participate in OSE wound repair following ovulation (Erickson and Shimasaki, 2003). Current theories suggest that most OvCas arise from the OSE (Auersperg et al., 2001). The OSE forms a monolayer of flat, cuboidal or low columnar epithelial cells on the ovarian surface separated from the underlying stroma by a basement membrane. The OSE functions to transport molecules such as growth factors, cytokines and hormones between the ovary and the peritoneal cavity, and participates in ovulation through the local production of proteases at the site of follicle rupture (Auersperg et al., 2001). In response to ovulation, OSE cells divide and migrate to seal the ovulatory wound. It is hypothesized that repeated cycles of wounding, proliferation and repair may contribute to accumulation of genetic damage and the development of OvCa (Fathalla, 1971). In the rat, it has been

demonstrated that coincident with ovulation, BMP4 is upregulated in OSE adjacent to the site of ovulation (Erickson and Shimasaki, 2003). This correlative data suggests that BMP4 may participate in OSE repair through promoting cellular division or migration. We have demonstrated that normal human OSE and OvCa cells possess an autocrine BMP signalling pathway (Shepherd and Nachtigal, 2003). BMP signalling can produce alterations in the behaviour of a diverse array of cancer cells, ranging from growth inhibition and apoptosis (Hjertner et al., 2001; Ide et al., 1997; Kawamura et al., 2002; Soda et al., 1998; Wach et al., 2001; Yamada et al., 1996) to influencing metastatic potential (Autzen et al., 1998; Dai et al., 2005; Hamdy et al., 1997; Raida et al., 2005). This report identifies differences in the BMP4 signalling response between normal OSE and OvCa cells. Previously we determined that target gene expression in response to BMP4 stimulation is more pronounced in OvCa cells versus normal OSE cells, and treatment with exogenous BMP4 can produce a cell spreading phenotype (Shepherd and Nachtigal, 2003). While BMP4 does not affect the growth rate of OvCa cells, BMP4-induced cell spreading resulted in decreased saturation density. Herein we examine the morphological and phenotypic consequences of BMP signalling in both primary human normal OSE and OvCa cells. BMP4-treated OvCa cells show changes in their behaviour including increased motility, adhesion, and invasion, and undergo a remodeling event resembling an EMT. EMT is associated with enhanced cellular motility and invasiveness (Huber et al., 2005; Thiery, 2002), which contributes to cancer cell transition through more aggressive stages. By contrast, normal OSE cells do not respond to BMP4 by altering cell motility, identifying an important difference between the response of normal OSE and OvCa cells to exogenous BMP4. We determined that the BMP inhibitor

Noggin could abrogate autocrine BMP4-induced activities, highlighting the contribution of BMP4 signalling in altering cellular behaviours. We have shown that the BMP4-induced changes in morphology and motility are dependent upon Smad signalling. Additionally, BMP4-induced EMT of OvCa cells correlates with reorganization of the actin cytoskeleton and activation of the Rho-GTPases. Collectively, these results indicate that BMP4 signalling may contribute to ovarian cancer progression.

4.3 MATERIALS AND METHODS

Culture of primary human OSE or OvCa cells was conducted as in section 2.1.1. Cells were treated with recombinant human BMP4 (R&D Systems) at a concentration of 10 ng/mL (278 pM) or noggin/Fc (R&D Systems) at a concentration of 100 ng/mL (1 nM). BMP4 and noggin/Fc were reconstituted in 0.1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS; vehicle). All experiments with OvCa cells were performed between passages 2 and 6.

Adenoviral cell transductions, fluorescence and confocal microscopy, as well as RNA isolation, QPCR and lentiviral cell transductions were performed according to sections 2.2, 2.3, 2.4, and 2.13 respectively.

Wounding assays were conducted as in section 2.5, where OvCa cells were seeded onto etched-grid coverslips, and pre-treated with vehicle, BMP4 (10 ng/mL), Ad-ALK3QD (MOI of 200), BMP4 (10 ng/mL) plus noggin/Fc (100 ng/mL) or noggin/Fc (100 ng/mL) alone for 48 h, after which the wounding assay was performed, and the cells replenished will full serum media containing the various treatments. Motility was then examined through phase contrast microscopy and digital imaging for a 12 h period post-wounding. Normal OSE cells were seeded onto 22 mm culture dishes pre-treated with vehicle, BMP4, BMP4 plus noggin/Fc, or noggin/Fc alone at the same concentrations for 48 h, and a wounding assay and subsequent analysis was performed as in section 2.5.

Cell area measurements were performed using phase-contrast microscopy and digital imaging along with the Image J analysis software. For each treatment condition, a minimum of 50 cells/image were measured, and 3 images/patient sample were assessed.

Unless otherwise stated, the data represent the compilation of cell area measurements from 3 different patient samples.

Adhesion assays were conducted as in section 2.6, where OvCa cells were pre-treated with vehicle, BMP4 (10 ng/mL), Ad-ALK3QD (MOI of 200), BMP4 (10 ng/mL) plus noggin/Fc (100 ng/mL) or noggin/Fc (100 ng/mL) alone for 48 h, after which the cells were radiolabelled for assessment of adhesion.

Invasion assays, re-plating and proliferation assays, Rho-GTPase activation and western analysis, as well as statistical analyses were performed as per sections 2.7, 2.8, 2.9 and 2.14, respectively.

Inhibition of Rho-GTPase activity was performed by pre-treating OvCa cells with either vehicle or BMP4 (10 ng/mL) for 48 h. 18 h before the wounding assay, OvCa cells were pre-treated with 10 μM of Y27632, a Rho-kinase (ROCK) inhibitor, or 100 μM of NSC23766, a Rac1 inhibitor (Calbiochem) O/N (approximately 18 h) in the presence or absence of BMP4 pre-treatment. Morphology was assessed through phase-contrast microscopy and digital imagery. A wounding assay was also subsequently performed as depicted in section 2.5, after which medium and/or treatments were replenished following production of the wound.

For the cofilin protein expression determinations, OvCa cells were incubated in low-serum conditions (0.2% FBS in complete growth medium) O/N, before stimulation with BMP4 (10 ng/mL) in low-serum conditions for 30 m, 1 h, 2 h, 4 h, 8 h and 24 h, after which total protein was isolated as per section 2.10. The same nitrocellulose membranes were utilized to determine p cofilin, total cofilin and GAPDH protein expression levels. After the p cofilin expression level was determined, the membranes

were stripped with a solution of 62.5 mM Tris pH 6.8, 2% SDS, and 100 mM β-mercaptoethanol for 15 m at 50°C. The membranes were then rinsed in TBST, and re-blocked in 5% milk/TBST for 2 h at RT prior to incubation with the anti-cofilin antibody. The expression of GAPDH was determined immediately after total cofilin measurements as outlined in section 2.11.4.

4.4 RESULTS

4.4.1 BMP4 Induces Cytoskeletal Re-Organization And Alters Morphology Of Normal OSE And OvCa Cells

To assess the change in normal OSE and OvCa morphology in response to BMP4, primary human cells were obtained from normal ovarian tissue biopsies or from ascites fluid samples originating from chemotherapy naïve OvCa patients. While the majority of OvCas are of the serous subtype, cells were obtained from patients diagnosed with serous, mucinous, and endometrioid subtypes and similar results were observed with cells from these different histological subtypes. OSE or OvCa cells were treated with 10 ng/mL recombinant human BMP4 for up to 7 days. BMP4 was selected for these studies because we had previously shown that BMP4 was expressed in human OSE and primary OvCa cells, whereas other BMP family members were rarely (BMP2) or not (BMP7) detected, and exogenous BMP4 produced changes in BMP target gene expression in these cells (Shepherd and Nachtigal, 2003). Phase contrast images show a distinct increase in cell size in response to BMP4 treatment in OSE and OvCa cells (Figure 4-1 and 4-2). On average, OSE and OvCa cells increase their cell area 2.8-fold in response to BMP4 treatment ($P < 0.0001$; OSE, 10 samples; OvCa, 18 samples). We had previously reported that BMP4 treatment did not change the general cytoskeletal architecture of primary ovarian cancer cells when assessed using anti-actin by indirect immunofluorescence (Shepherd and Nachtigal, 2003). To refine these previous analyses, we switched our technique to use Phalloidin, which is designed to specifically detect filamentous actin alone, in combination with confocal microscopy, as opposed to using indirect immunofluorescence and the anti-actin antibody that detects both globular and

filamentous actin. The experiments conducted using Phalloidin have allowed us to more clearly visualize the alterations in the actin cytoskeleton. Herein we show a reorganization of the actin cytoskeleton from the predominance of cortical actin into actin stress fibers throughout the cell in response to BMP4 treatment in both normal OSE and OvCa cells. In the previous experiments, our analyses using anti-actin likely masked this effect because of the high level of globular actin present in the cells, and the high level of background staining. Reorganization of filamentous actin is characteristic of a cell spreading response (Soranno and Bell, 1982). Cell spreading requires changes in adhesion to the ECM (Pierres et al., 2002; Pierres et al., 2003; Raz et al., 1986). Vinculin is a focal adhesion protein (FAP) that nucleates many molecular components, including F-actin, which is required for cellular motility and adhesion. BMP4 treatment induced a re-localization of vinculin to membrane ruffles and co-localized with the termini of F-actin stress fibers in OvCa cells (Figure 4-2, VIII). Colocalization of vinculin and F-actin does not occur in the vehicle treated cells (Figure 4-2, IV). F-actin and vinculin co-localization suggests that these molecules form a complex that participates in cellular remodeling and enhanced adhesion and motility. To further evaluate the contribution of BMP signalling to morphological changes, OvCa cells were transduced with an adenovirus expressing a constitutively activated BMP Type IA receptor, ALK3QD. ALK3 was selected for these studies because we had determined that the BMP Type IB receptor (ALK6), which is also capable of transmitting BMP4 signals, is rarely expressed in OSE or OvCa cells (Shepherd and Nachtigal, 2003). These analyses were conducted only in OvCa cells due to the limited availability of normal OSE cells; OSE cells were used for assays requiring fewer cells. Compared to non-transduced (NT; Figure 4-3, I-

IV) OvCa cells, Ad-ALK3QD transduced cells showed consistent changes in morphology and cell area after transduction (5-fold increase in area, $P < 0.0004$; Figure 4-3, V-VIII). To control for adenoviral transduction and protein overexpression, OvCa cells were transduced with Ad-GFP (Figure 4-3, IX-XII); these cells did not exhibit morphological alterations compared to NT cells. ALK3QD expression was detected by western analysis (Figure 4-3, XIII). For transduction experiments, a multiplicity of infection (MOI) of 200 was selected because it produces approximately 100% OvCa cell transduction efficiency.

4.4.2 BMP4 Increases mRNA Expression Of Integrins, FAPs And ECM Proteins

To assess the mRNA expression of a subset of molecules that control cell morphology, adhesion, and migration, QPCR analysis was performed on total RNA isolated from cultures of OvCa cells treated from 1 to 7 days with vehicle or 10 ng/mL of BMP4. An increase in the mRNA of many integrin subunits, FAPs and ECM proteins in response to BMP4 treatment was observed at different time points (Table 4.1). The mRNA for ECM proteins was increased at earlier times post-BMP4 treatment, while the mRNA for integrin receptors and FAPs were only increased after several days of continuous BMP4 treatment. These data show that BMP4 can induce alterations in the expression of molecules that participate in cellular interaction with the extracellular environment.

4.4.3 BMP4 Induces An EMT Response In OvCa Cells

The morphological response of primary OvCa cells to BMP4 treatment resembles an EMT. In response to BMP4 treatment, the staining pattern for αSMA was altered and persisted for up to 8 d (Figure 4-4). αSMA distribution was similar to the staining pattern observed in mesenchymal cell types (Kowanetz et al., 2004). OvCa cells produced a

committed morphological change in response to one treatment of BMP4 for 48 h (Figure 4-5). These cells continued to proliferate after undergoing morphological alterations, were efficiently lifted and re-plated (~83% average re-plating efficiency for vehicle or BMP4 treated cells), and maintained their mesenchymal morphology after re-plating (Figure 4-6). Additionally, mRNA levels for the EMT markers *Snail* and *Slug* increased an average of 2 fold as early as 1 h after BMP4 treatment (Figure 4-7). Changes in mRNA preceded increased Snail and Slug protein levels (Figure 4-8). By contrast, mRNA for the epithelial marker *E-cadherin* was not altered initially in the presence of BMP4; however, mRNA and protein levels decreased (~50% compared to untreated cells) by 48 h and persisted at lower levels until the experiment was terminated (Figures 4-7 and 4-8). These results are consistent with transcriptional repression of E- cadherin by Snail and Slug (Batlle et al., 2000). Taken together, these data strongly suggest that exposure to exogenous BMP4 can induce an EMT in primary OvCa cells.

4.4.4 OvCa Cell Adhesion (Reattachment) Is Increased In Response To BMP4

To functionally assess the ability of OvCa cells to interact with various ECM substrates, OvCa cells (pre-treated with vehicle or BMP4 for 48 h) were plated on culture dishes coated with control (1% BSA in PBS) or various ECM substrates and adhesion assays were performed. BMP4 induced a significant increase in cell adhesion, and this was the case for all of the ECM coatings tested (Figure 4-9). OvCa cells also showed selective interaction with different ECM substrates, as indicated by the highest adhesion on fibronectin and the lowest adhesion on laminin (Figure 4-9 B, C, D). Additionally, while vehicle treated OvCa cells appear to have similar morphologies when cultured on glass, collagen I, collagen IV, or fibronectin, these cells have an altered morphology

when cultured on laminin (Figure 4-10). These results suggest that OvCa cells do not adhere or interact with all ECM substrates in the same manner. In response to BMP4, OvCa cells display a cell spreading phenotype when cultured upon all ECM substrates including laminin. Transduction of OvCa cell samples with Ad-ALK3QD also significantly increased cell adherence to all coatings tested (Figure 4-11). To determine if inhibition of BMP4 signalling would affect cell adherence, cells were also co-treated with either BMP4 plus the BMP inhibitor noggin/Fc (100 ng/mL; (Groppe et al., 2002) or noggin/Fc alone for 48 h prior to assessing reattachment. Treatment with BMP4 plus noggin/Fc or noggin/Fc alone significantly reduced cell reattachment to below control (vehicle) levels on coating control, collagen I, and collagen IV, suggesting that noggin/Fc is inhibiting autocrine BMP signalling activity that may regulate basal adhesive protein levels (Figure 4-11).

4.4.5 BMP4 Increases OvCa Cell Motility But Not Normal OSE Motility

A wounding assay was used to examine cell motility of OvCa cells in response to BMP4. OvCa cells were treated with vehicle or BMP4 for 48 h prior to producing a 1 mm wound. The ability of these cells to close the wound was then assessed over time. Over a period of 12 h the BMP4-treated cells migrated to cover a greater surface area of the wound as compared to vehicle treated cells (Figure 4-12). A significant increase in the percentage of the total area covered by cells that were subjected to BMP4 treatment was apparent 6 h after producing the wound (Figure 4-12). At 12 h the average increase in motility for BMP4-treated samples is a 2 fold increase ($P < 0.0005$). Transduction of OvCa cells with Ad-ALK3QD also showed a significant increase in cell motility (Figure 4-13). In addition, noggin/Fc abrogated cell motility induced by BMP4 treatment, and

treatment of OvCa cells with noggin/Fc alone decreased cell migration to below control (vehicle) levels. These data support the existence of an autocrine BMP signalling pathway that is capable of promoting OvCa cell motility. To evaluate the motility response in normal OSE cells, wounding assays were conducted on 9 different normal OSE cell samples. Despite the fact that normal OSE respond to BMP4 by altering cell morphology (Figure 4-1) and alter BMP4 target gene expression (Shepherd and Nachtigal, 2003), we found no significant difference in motility between the control (vehicle) cells and any of the other treatments (Figure 4-14, A, B). These results show that the motility of normal OSE cells was not affected by BMP4 or noggin/Fc in the same manner as OvCa cells, suggesting that OvCa cells have acquired the ability to enhance motility in response to BMP4.

4.4.6 BMP4 Treatment Or ALK3QD Expression Increases OvCa Cell Invasion

We also examined the effect of BMP4 ligand on OvCa cell invasive capabilities through a collagen I matrix. We found that in response to BMP4 invasion was increased when naïve cells from three different patient samples were exposed to BMP4 during the invasion assay over an 18 h time period (Figure 4-15 A, B). BMP4 pretreatment (48 h) of these same primary OvCa cells produced different results; only one patient sample exhibited a significant increase in invasion, whereas no significant change was observed in two other patient samples. Further investigation would be required to determine why there is a differential effect on invasion in the two patient samples that do not respond to BMP4 pretreatment, whereas the third patient sample maintained sensitivity. BMP receptor downregulation is one possible explanation. These results are in contrast to a statistically significant increase in invasion when OvCa cells are transduced with an

expression vector for a constitutively-active BMP type I receptor (ALK3QD). Six different OvCa patient samples were tested and all showed significant increases in invasion over NT or Ad-GFP transduced cells (Figure 4-15 C, D). No significant difference in invasion was seen between the NT and Ad-GFP transduced OvCa samples. Furthermore, we conducted a modified Boyden chamber analysis using Transwell inserts (Richard et al., 2006) to determine whether BMP4 was chemotactic. Under no circumstances did BMP4 induce directional migration of primary OvCa cells (data not shown). Although invasion was assessed through a simplified gel matrix composed of a single ECM protein (collagen I), these results clearly indicate that BMP4 signalling contributes to increased invasive capabilities of OvCa cells.

4.4.7 BMP4 Treatment Results In Increased Rho-GTPase Activation

Alterations in the actin cytoskeleton, motility, adhesion, and invasion typically involve activation of the Rho GTPases (Hall, 1998; Ridley, 2001; Schmitz et al., 2000). To investigate whether BMP4 treatment induced Rho-GTPase activation, Rac1, Rho and Cdc42 pulldown assays were performed. These assays measure levels of activated, GTPbound, Rho-GTPases. In comparison to vehicle treated cells, BMP4-treated cells show consistently higher levels of activated Rac1, Rho and Cdc42 between 1-4 h after treatment, and this activation decreased to baseline levels by 24 h (Figure 4-16). To determine whether the BMP4-induced increases in cellular motility were dependent upon Rho-GTPase activity, OvCa cells were pre-treated O/N with specific inhibitors of Rho kinase, (ROCK; Y27632) and Rac1 (NSC23766) activity, and cellular morphology and motility was assessed in the presence or absence of BMP4 pre-treatment for 48 h. ROCK and Rac1 inhibitors significantly modified OvCa cellular morphology, exhibited by the

formation of cytoplasmic projections and this morphologic change was not affected by the presence of BMP4 (Figure 4-17, A). Inhibition of ROCK or Rac1 activity also resulted in the significant reduction of cellular motility even in the absence of BMP4 stimulation (Figure 4-17, B), demonstrating that both Rho and Rac1 activity are most likely required in order for OvCa cells to induce morphological alterations in response to BMP4, and for OvCa cells to become motile. These results indicate that BMP4 signalling induced the activation of the small GTPases Rac1, Rho and Cdc42 in primary OvCa cells, and that GTPase activity is strongly involved OvCa cellular motility, however the precise mechanism of Rho-GTPase activation mediated by the BMP4 signalling pathway remains to be determined.

4.4.8 BMP4-Induced Changes In OvCa Cell Morphology And Motility Are Smad-Dependent

The canonical BMP signal transduction pathway consists of BMPs signalling through a complex of type I and type II serine/threonine kinase membrane receptors, subsequently activating the intracellular Smads 1, 5, and 8. These R-Smads then associate with the common Smad 4, and translocate to the nucleus to affect target gene expression (Attisano and Wrana, 2002). Recent evidence has shown that BMP signalling can be transduced through Smad-independent mediators such as Cdc42 and LIMK1 (Foletta et al., 2003; Lee-Hoeflich et al., 2004) through interaction with the type II BMP receptor, BMPRII. We tested the activity of LIMK in response to BMP4 treatment through the phosphorylation status of its downstream effector cofilin, and determined that there was no alteration in cofilin phosphorylation in response to BMP4 treatment (Figure 4-18). We did not determine if Cdc42 interacted with BMPRII in OvCa cells in response

to BMP signalling. We did evaluate whether blocking Smad protein expression would affect the BMP4-induced changes in phenotype seen in OvCa cells. Lentiviruses expressing inhibitory shRNA molecules targeting the mRNA degradation of Smads 1 and 5 (BMP-specific Smads) as well as Smad 4 (common Smad) were employed to transduce OvCa cells. A scrambled lentivirus expressing a shRNA molecule with no known RNA target was also employed as a lentivirus expression control (Table 2-2). Smad protein expression was measured in response to lentivirus transduction, and all lentiviruses tested except for the Scrambled lentivirus decreased target protein expression to at least 50% below control levels (Figure 4-19). Furthermore, the Scrambled lentivirus had no effect on Smad 1, 4 or 5 protein expression levels. Two Smad 4 shRNA constructs (Smad 4#1 and Smad 4#2) were engineered to target the 3' (Smad 4#1) and 5' (Smad 4#2) regions of the human Smad 4 mRNA, and when transduced in OvCa cells, produced similar knockdown in Smad 4 protein expression (Figure 4-19 A and B). Furthermore, in the presence of the Smad lentivirus shRNA constructs BMP4 had a reduced capacity to induce Id1 protein expression (Figure 4-20). These results indicate that targeting Smad mRNA degradation through the lentiviral transduction of shRNA molecules results in effective decrease in Smad protein expression as well as downstream target Id1 mRNA and protein expression.

To assess whether the decrease in Smad protein expression translated into a functional change of cellular behaviours in response to BMP4 signalling, OvCa cells were transduced with Smad lentivirus shRNAs and cellular morphology was assessed over a 4 d period in response to BMP4. While Smad lentivirus transduction had no effect on cellular morphology (Figure 4-21), BMP4 induced a morphologic change in

untransduced and Scramble-transduced cells, characterized by cell spreading and increases in cellular size. However when OvCa cells were transduced with the Smad lentiviruses (targeting Smads 1, 4, and 5), the BMP4-induced morphologic change was almost completely blocked after 4 d of BMP4 treatment (Figure 4-21). Additionally, expression of the Smad lentiviruses completely blocked cellular motility induced by BMP4 treatment (Figure 4-22) as compared to the Scrambled lentivirus. These results clearly indicate that Smad 1, 4 and 5 expression are required for BMP4 signalling to exert its changes on OvCa cellular behaviours, and contribute to the idea that cellular remodeling in response to BMP4 signalling is Smad-dependent.

4.5 DISCUSSION

We determined that BMP4 signalling is able to affect cellular behaviours in primary human OvCa cells, including changes in morphology, adhesion, motility, and invasion. BMP4 treatment of OvCa and normal OSE cells induces a significant increase in cell area in parallel with cytoskeletal reorganization. Interestingly, the BMP4-induced change in OvCa cell morphology is coincident with changes in markers associated with EMT. Furthermore, while BMP4 increases OvCa cell motility, normal OSE cell motility is not enhanced by treatment with exogenous BMP4. We also observed changes in the level of activated Rho-GTPases (Rho, Rac1, Cdc42) in OvCa cells treated with BMP4. These data argue that BMP signalling alters OvCa cellular behaviours that may promote OvCa metastatic progression.

BMP4 treatment of normal OSE and OvCa cells induces reorganization of the actin cytoskeleton into stress fibers throughout the cell, and increases co-localization of F-actin with vinculin to membrane ruffles. Our data is consistent with BMP4 signalling causing a remodeling of the cellular machinery that is responsible for cellular morphology, adhesion, motility and invasion (Hall, 1998; Ridley, 2001; Schmitz et al., 2000). BMP4 stimulation of OvCa cells changes the mRNA expression for various integrin receptors, ECM proteins and FAPs. Differential expression of adhesive proteins on OvCa cells in response to BMP4 likely mediates the increased adhesion and interaction with various ECM coatings. Blocking autocrine BMP4 signalling reduces OvCa cell adhesive properties, suggesting that endogenous BMP4 activity is important for cell-ECM interaction. However, this BMP4-induced adhesion varies depending on the type of extracellular stimulus that is provided. OvCa cells showed the highest

adhesion on fibronectin, and the lowest adhesion on laminin. The finding that adhesion on laminin was similar to adhesion on the coating control even in the presence of BMP4, and that OvCa cell morphology was different on a laminin substrate suggests that OvCa cells may show limited interactions with this ECM protein. Normal OSE cells rest on a basement membrane composed of various ECM proteins including laminin (Auersperg et al., 2001), and the fact that OvCa cells may show tenuous attachment to laminin indicates that OvCa cells have acquired this property in order to facilitate detachment from its basement membrane during the metastatic progression of OvCa. OvCa metastasis is not only characterized by OvCa cell exfoliation from the primary tumor and diffusion throughout the peritoneal cavity, but also by attaching and establishing micrometastases on peritoneal surfaces (Robert E. Scully, 1998). Our results suggest that BMP4 affects the cells ability to regulate adhesion proteins that allow them to adhere to diverse strata, which would be beneficial for OvCa metastatic progression.

BMP4-induced morphological changes point to the possibility that OvCa cells can be remodeled to exhibit mesenchymal-like phenotype. We observed that OvCa cells increase cellular motility and invasive capabilities through a collagen I matrix in response to BMP4; these behaviours are associated with EMT (Huber et al., 2005; Thiery, 2002). In correlation with these altered cellular behaviours, we found that mRNA and protein levels of Snail and Slug are elevated shortly after BMP4 treatment, while E-cadherin mRNA and protein levels decrease following 48 h of BMP4 treatment. Ectopic overexpression of Snail and Slug in the SkOV3 human ovarian cancer cell line enhances their motility, invasiveness and tumorigenicity (Kurrey et al., 2005). In addition, we demonstrated that primary OvCa cells express αSMA and its immunolocalization was

altered to resemble a mesenchymal pattern after BMP4 treatment. These features show that additional mesenchymal cell characteristics are acquired over a 48 h period in response to exogenous BMP4 treatment. Overexpression of ALK3QD results in similar morphological and behavioural changes as seen with exogenous BMP4 treatment. These results suggest that BMP4-induced effects are most likely produced by signalling through ALK3. Moreover, the rapid induction and increased magnitude of the cellular responses induced through ALK3QD is likely a reflection of the presence of a constitutively active receptor versus reliance on endogenous receptor dynamics that transmit signals with regulated amplitude. Case in point, primary OvCa cell responses to exogenous BMP4 ligand are not universal; a small proportion (5-10%) of the OvCa cells observed do not exhibit BMP4-induced changes in morphology, suggesting that variations in endogenous BMP receptor signalling in OvCa cells may account for the variation in morphologic responses. This is in contrast to the response that is produced by constitutive ALK3 signalling which is evidenced by a morphologic change in 100% of OvCa cells. Similarly, exogenous BMP4 may produce EMT due to a higher level of signalling activity compared with autocrine BMP4 signalling, suggesting that OvCa cells require a certain threshold of signalling activity in order to induce EMT *in vitro*. A putative function for autocrine BMP4 in OvCa cells was illustrated through blocking endogenous BMP4 signalling. Treatment of OvCa cells with noggin/Fc alone decreased both adhesion and motility in many cases to below control levels. These results are in agreement with the findings from Moll *et al* who demonstrated that transfecting established OvCa cell lines with an expression vector for the BMP inhibitor chordin, or using conditioned medium containing noggin, reduced cell motility and their ability to

invade Matrigel (Moll et al., 2006). Together, these data support the argument that autocrine BMP4 may contribute to OvCa cell metastasis through enhancing motility and adhesion. Surprisingly, normal OSE motility is not enhanced by treatment with exogenous BMP4 nor is it abrogated by noggin/Fc alone. This observation complements our previous finding showing that select BMP4 target gene expression, e.g. *ID1* and *ID3* proto-oncogene, is more pronounced in OvCa cells compared to OSE cells (Shepherd and Nachtigal, 2003). The molecular basis of these differential responses remain unknown; however, we have determined that the mRNA for BMPRII and ALK3, and Smad1 and Smad5 protein levels remain unchanged between OSE and OvCa cells (BLT, TGS, MWN, unpublished observations). Collectively, these data suggest that a component of the BMP4 signalling pathway, an interacting signalling pathway, or a downstream effector molecule(s) is altered in OvCa cells. These distinct cellular responses begin to provide insight into how autocrine BMP4 may contribute to OvCa etiology.

BMP signalling is classically mediated via a Smad cascade that is activated by the BMP receptor complex. Recent studies have demonstrated that BMP signalling can also be mediated through alternative intracellular mediators, including Cdc42 and LIMK 1 (Foletta et al., 2003; Lee-Hoeflich et al., 2004). Rho-GTPases act upstream of LIMKs to regulate actin polymerization. While the pathway leading from the activated BMP receptor complex to Cdc42 remains undetermined, these investigators have identified a direct interaction of LIMK1 with the C-terminal tail of BMPRII. LIMK1 is activated by Cdc42 following BMP activation of the BMP receptor complex. Activated LIMK1 remains associated with the activated BMP receptor complex, and is capable of phosphorylating its downstream effector cofilin. Cofilin is a member of the F-actin

binding protein family that regulates actin dynamics. We show that Cdc42, along with Rac1 and Rho, are activated shortly after BMP4 treatment of OvCa cells, which precedes the observed morphological changes associated with actin remodeling. Interestingly, elevated Rho expression has been correlated with more aggressive OvCa behaviour (Horiuchi et al., 2003a; Kleer et al., 2005). In agreement with Lee-Hoeflich *et al*, our results point to the role of BMP signalling in activating modulators of actin cytoskeletal dynamics, the Rho-GTPases. In light of these results, we investigated whether LIMK activity in OvCa cells was altered in response to BMP4 stimulation through its downstream effector, cofilin. In experiments using cell lysates from three patient samples we measured LIMK1 activity in BMP4 treated OvCa cells from 30 m to 24 h and determined that there was no significant alteration in cofilin phosphorylation. Moreover, the phenotypic responses of primary OvCa cells to BMP4 treatment can be replicated using an activated type I receptor, ALK3QD, which signals independent of the BMPRII. To our knowledge there is no evidence that LIMK1 interacts with BMP type I receptors. Therefore, these data suggest that LIMK1 is unlikely to participate in the BMP4-induced cellular responses in OvCa cells, and that BMP4-induced cellular remodeling may not be induced by Smad-independent signalling. To support this assumption, we used lentiviruses that express shRNA molecules targeting the mRNA of Smads 1, 4 and 5, and assessed cellular morphology and motility in response to BMP4 treatment. The knockdown of Smad 1, 4 and 5 expression partially blocked the induction of the BMP target protein Id1, partially blocked the changes in morphology induced by BMP4, as well as completely blocked the BMP4-induced increase in motility. It remains to be determined whether alterations in small Rho-GTPase activation are still induced after

Smad1, 4, and 5 knockdown, which would provide evidence to uncover the role that Smad-dependent versus Smad-independent signalling may play in Rho-GTPase activation and alteration of cellular behaviours. Taken together, these results indicate that the BMP4-induced changes in the signalling, morphology and motility of OvCa cells are in part a result of Smad-dependent signalling.

We observe that Rho-GTPase activation peaks between 1-4 h after a single treatment with BMP4 and returns to baseline levels of activity by 24 h; however, BMP4-induced alterations in cell morphology, adhesion, motility and invasive capabilities are observed after ~48 h. Additional experiments were conducted using three different patient samples for up to 72 h post-BMP4 treatment. In all cases Rho-GTPase activation had returned to baseline by 24 h and remained at baseline levels up to 72 h after a single treatment with BMP4 (data not shown). We hypothesize that the transient increase in Rho-GTPase activity is one of the initiating events leading to cellular remodeling resulting in cells possessing these altered traits. Furthermore, enhanced migration was only observed following BMP4-induced morphological alterations. Indeed, no increases in cell motility above control levels was observed when naïve OvCa cells were treated with BMP4, or when OvCa cells were pretreated with BMP4 for 24 h–prior to an observed change in cellular morphology (data not shown). These data show that despite small Rho-GTPase activation in response to BMP4 initially, this event alone is insufficient to augment motility rates in naïve OvCa cells. Therefore, morphological alterations appear to be a prerequisite for enhanced motility, which is consistent with BMP4 inducing an EMT in OvCa cells (Barrallo-Gimeno and Nieto, 2005). Rho-GTPases are well known to participate in cellular processes of adhesion, motility and

invasion (Barrallo-Gimeno and Nieto, 2005; Savagner, 2001). While we believe that Rho-GTPases likely play a part in the cellular responses that we have presented, our experimental conditions preclude measuring Rho-GTPase activity during the assessment of cellular motility and adhesion. This is due to the fact that small Rho-GTPase activation assays are conducted under low serum conditions (0.2% FBS) in order to increase the sensitivity of the assay. In our experience, assessment of Rho-GTPase activation when cells are maintained in high serum (10% FBS) produces a high background level of activation. Under normal growth conditions BMP4 pretreatment promotes enhanced adhesion and migration, but given that these experiments are conducted in the presence of 10% FBS, we cannot directly determine if Rho-GTPase activation is also increased under these experimental conditions. However, use of the Rho kinase inhibitor Y-27632 and the Rac1 inhibitor NSC23766 in the presence or absence of BMP4 under normal growth conditions resulted in the significant reduction of cellular motility, strongly suggesting that the Rho-GTPases are involved in this activity. BMP4 responses are dependent upon Smad signalling, however it remains to be determined how other mediators such as the Rho-GTPases are connected to the BMP4 signalling cascade. BMP4 signalling affects a wide variety of cellular behaviours in OvCa cells, leading us to postulate that BMP4 signalling has the ability to promote altered OvCa cell adhesion, motility and invasion which may translate into increased metastatic potential and poor patient outcomes. Overall, our studies suggest a link between autocrine BMP signalling mediated through Smad-dependent signalling, the Rho-GTPase family, and Snail and Slug-induced EMT that collectively contribute to enhanced OvCa cell adhesion, motility, and invasiveness. The fact that BMP4-induced motility and adhesion can be blocked by anti-Smad shRNA,

and with a BMP signalling inhibitor (noggin) provides a rationale to develop and test anti-BMP therapeutics (Chen et al., 2002; Haudenschild et al., 2004) to prevent or stop OvCa progression.

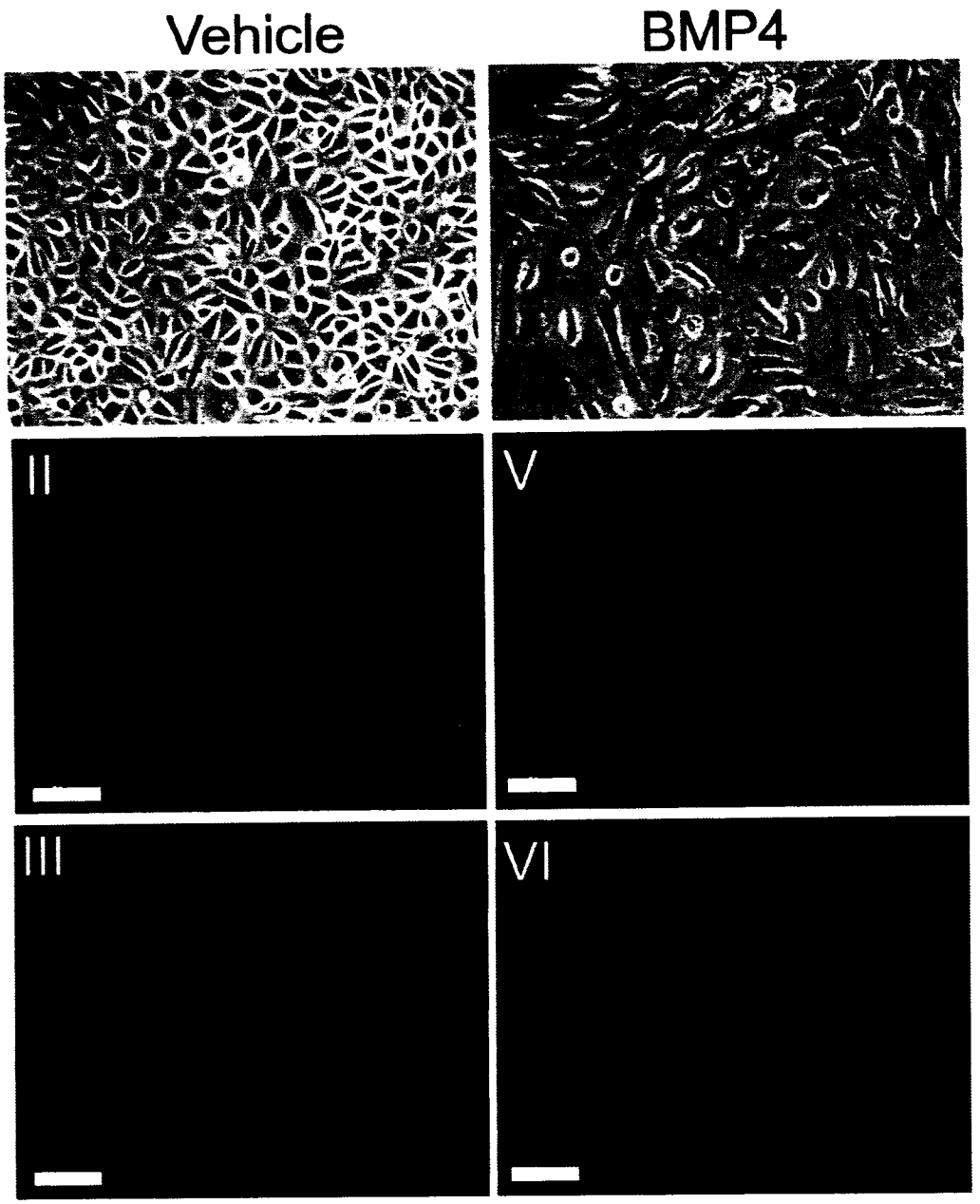


Figure 4-1. BMP4 signalling induces cytoskeletal re-organization and alters morphology of normal OSE cells. Normal OSE cells (I-VI) treated with vehicle of BMP4 (10 ng/mL). (I and IV) Cells were visualized by phase-contrast microscopy. Magnification x 200. (II and V) OSE cells stained with Alexa 488-conjugated phalloidin to reveal filamentous actin stress fibers (green), visualized by fluorescence microscopy. (III and VI) OSE cells stained with anti-vinculin plus Alexa 488-conjugated secondary antibodies (green), visualized through fluorescence microscopy. (II, III, V and VI) Samples were co-stained with Hoechst 33258 to reveal nuclei (blue). Similar results were observed using primary cells from 10 normal OSE samples. Bar, 50 μm.

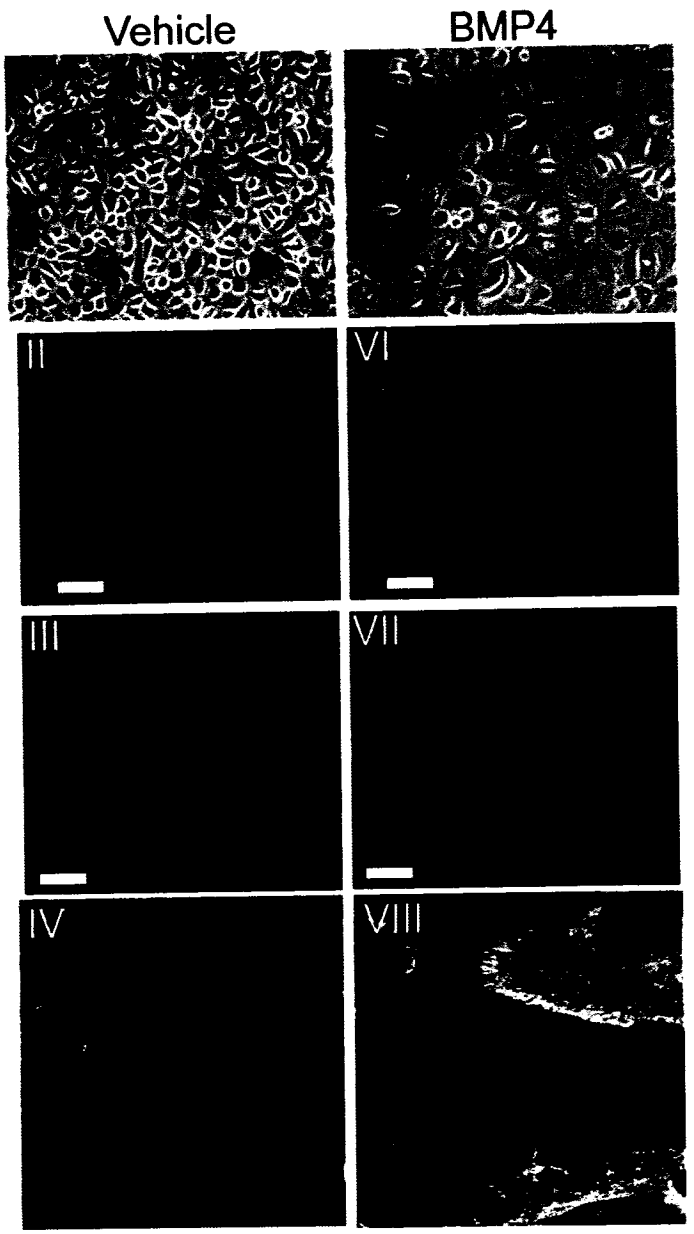


Figure 4-2. BMP4 signalling induces cytoskeletal re-organization and alters morphology of OvCa cells. OvCa cells treated with vehicle or BMP4 (10 ng/mL). (I and V) Cells were visualized by phase-contrast microscopy. Magnification x 200. (II and VI) OvCa cells were stained with Alexa 488-conjugated phalloidin to reveal filamentous actin stress fibers (green), visualized through fluorescence microscopy. (III and VII) OvCa cells stained with anti-vinculin plus Alexa 488-conjugated secondary antibodies (green), visualized through fluorescence microscopy. (IV and VIII) OvCa cells were co-stained with rhodamine-conjugated phalloidin (red) and anti-vinculin plus Alexa 488-conjugated secondary antibodies (green), visualized through confocal microscopy. Magnification x 630. Co-localized protein appears yellow. Similar results were observed using primary cells from 18 OvCa samples. Bar, 50 μm.

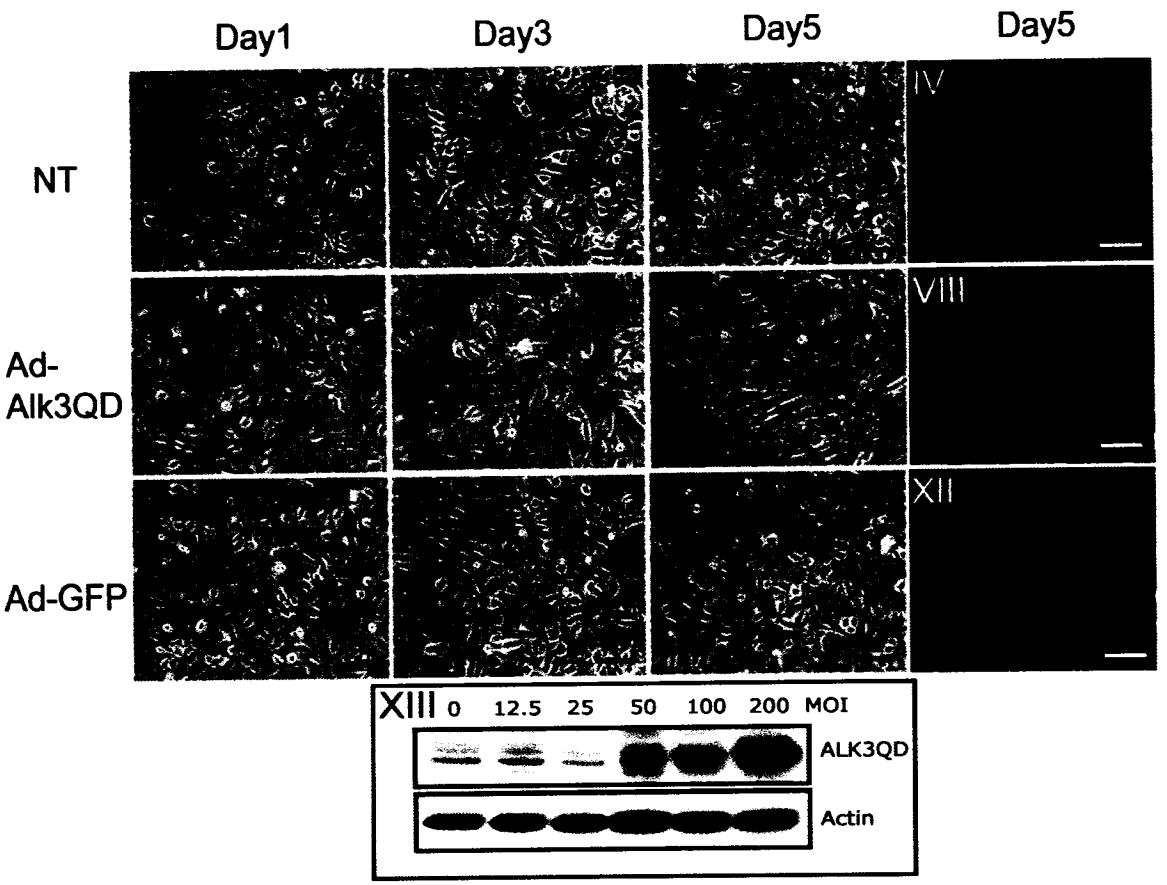


Figure 4-3. ALK3QD overexpression alters the morphology of OvCa cells. The morphology of non-transduced (NT) OvCa cells (I-IV), OvCa cells transduced with Ad-ALK3QD (V-VIII) or transduced with Ad-GFP (IX-XII) was visualized over 5 days by phase-constrast microscopy. (IV, VIII and XII) On day 5, F-actin was visualized using rhodamine-phalloidin staining (red) and fluorescence microscopy; samples were co-stained with Hoechst 33258 to reveal nuclei (blue). Similar results were obtained using primary cells from five OvCa samples. (XIII) Increasing multiplicity of infection of Ad-ALK3QD was used to transduce primary OvCa cells. ALK3QD was detected by western analysis; actin was examined to assess protein loading. Bar, 50 μm.

A	INTEGRINS							
Day	alpha 1	alpha 2	alpha 3	alpha 4	alpha 5	alpha V	beta 1	beta 3
1	1.14 +/- 0.06 (0.39)	1.01 +/- 0.1 (0.43)	1.17 +/- 0.16 (0.42)	1.46 +/- 0.59 (0.07)	1.29 +/- 0.39 (0.09)	1.34 +/- 0.7 (0.17)	1.04 +/- 0.24 (0.38)	1.34 +/- 0.52 (0.11)
2	1.23 +/- 0.33 (0.09)	1.13 +/- 0.4 (0.25)	1.37 +/- 0.37 (0.44)	1.38 +/- 0.42 (0.06)	2.11 +/- 1.35 (0.07)	1.33 +/- 0.48 (0.09)	1.08 +/- 1.15 (0.30)	1.34 +/- 0.41 (0.07)
3	0.89 +/- 0.09 (0.32)	0.93 +/- 0.13 (0.14)	1.03 +/- 0.29 (0.41)	1.2 +/- 0.28 (0.21)	0.83 +/- 0.27 (0.12)	0.98 +/- 0.43 (0.46)	2.12 +/- 2.53 (0.19)	1.21 +/- 0.28 (0.09)
4	1.58 +/- 1.18 (0.17)	1.61 +/- 1.28 (0.17)	0.96 +/- 0.25 (0.37)	1.18 +/- 0.18 (0.06)	1.3 +/- 0.8 (0.23)	0.89 +/- 0.22 (0.16)	1.69 +/- 1.36 (0.16)	1.35 +/- 0.85 (0.21)
5	1.07 +/- 0.25 (0.28)	0.98 +/- 0.04 (0.16)	0.98 +/- 0.23 (0.43)	1.2 +/- 0.72 (0.28)	1.3 +/- 0.4 (0.08)	1.27 +/- 0.26 (0.06)	1.74 +/- 1.28 (0.13)	1.07 +/- 0.32 (0.31)
6	1.81 +/- 1.46 (0.14)	1.62 +/- 1.18 (0.15)	1.14 +/- 0.26 (0.14)	1.24 +/- 0.49 (0.17)	2.08 +/- 0.48 (0.003)	1 +/- 0.23 (0.48)	1.72 +/- 0.36 (0.006)	1.86 +/- 0.53 (0.01)
7	1.87 +/- 0.30 (0.001)	2.01 +/- 0.39 (0.002)	0.9 +/- 0.34 (0.28)	1.86 +/- 0.45 (0.006)	2.39 +/- 0.84 (0.01)	1.85 +/- 0.39 (0.003)	2.12 +/- 0.79 (0.02)	2.14 +/- 0.69 (0.01)

B	ECM PROTEINS				
Day	col1	col4	laminin	fibronectin	vitronectin
1	1.62 +/- 0.42 (0.01)	2.14 +/- 0.78 (0.015)	1.2 +/- 0.54 (0.26)	1.25 +/- 0.24 (0.06)	0.96 +/- 0.18 (0.34)
2	1.76 +/- 0.46 (0.01)	0.96 +/- 0.26 (0.39)	1.22 +/- 0.57 (0.25)	1.25 +/- 0.35 (0.12)	1.03 +/- 0.35 (0.42)
3	1.15 +/- 0.10 (0.14)	0.7 +/- 0.22 (0.19)	1.23 +/- 0.57 (0.23)	1.66 +/- 0.63 (0.06)	0.95 +/- 0.41 (0.42)
4	1.13 +/- 0.29 (0.19)	0.68 +/- 0.21 (0.28)	3.26 +/- 0.8 (0.006)	3.83 +/- 1.81 (0.03)	0.94 +/- 0.39 (0.39)
5	1.75 +/- 0.42 (0.008)	1.99 +/- 0.51 (0.006)	1.04 +/- 0.34 (0.41)	1.99 +/- 0.18 (0.0008)	0.88 +/- 0.28 (0.23)
6	2.49 +/- 0.8 (0.007)	2.27 +/- 1.06 (0.03)	1.83 +/- 0.71 (0.05)	2.8 +/- 1.02 (0.02)	1.04 +/- 0.32 (0.41)
7	1.84 +/- 0.35 (0.003)	2.78 +/- 1.55 (0.03)	1.68 +/- 0.49 (0.03)	2.52 +/- 1.2 (0.03)	1.19 +/- 0.28 (0.13)

C	FAPs and ABP			
Day	vinculin	talin	fak	filamin
1	1.19+/- 0.29 (0.14)	1.85 +/- 0.36 (0.009)	1.02 +/- 0.27 (0.45)	3.19 +/- 1.03 (0.01)
2	1.09 +/- 0.35 (0.32)	1.33 +/- 0.62 (0.18)	1.27 +/- 0.33 (0.1)	2.71 +/- 0.75 (0.01)
3	0.81 +/- 0.29 (0.15)	2.45 +/- 0.76 (0.02)	1.94 +/- 0.52 (0.03)	0.97 +/- 0.43 (0.46)
4	0.91 +/- 0.29 (0.28)	1.39 +/- 1.21 (0.28)	1.56 +/- 0.9 (0.02)	2.55 +/- 1.1 (0.03)
5	1.01 +/- 0.24 (0.46)	0.91 +/- 0.34 (0.35)	0.84 +/- 0.65 (0.33)	2.42 +/- 0.74 (0.02)
6	1.27 +/- 0.36 (0.12)	1.53 +/- 0.62 (0.09)	1.18 +/- 0.65 (0.30)	2.12 +/- 0.92 (0.04)
7	1.12+/- 0.28 (0.21)	0.96 +/- 0.2 (0.35)	1.19 +/- 0.43 (0.24)	3.46 +/- 1.21 (0.01)

Table 4-1. QPCR assessment of integrins, ECM, FAP, and ABP expression in OvCa cells. OvCa cells were treated with 10 ng/mL of BMP4 and total RNA was isolated every day for a period of 7 days. **(A)** Fold change in mRNA expression of integrins α 1, 2, 3, 4, 5 and V, β 1 and 3, is shown in response to BMP4 treatment, relative to vehicle-treated cells. Data is shown as fold change in expression +/- standard deviation between BMP4 treated relative to vehicle treated cells. The number in brackets is the *P* value for each molecule. **(B)** Fold change in mRNA expression of ECM proteins collagen 1 (col 1) and collagen 4 (col 4), laminin, fibronectin and vitronectin in response to BMP4 treatment. **(C)** Fold change in mRNA expression of the focal adhesion proteins (FAPs) vinculin, talin and focal adhesion kinase (FAK), as well as the actin-binding protein (ABP) filamin in response to BMP4 treatment. Data was obtained using 11 OvCa patient samples from a minimum of three independent experiments; duplicate measurements were made for each sample within each experiment. A significant change in mRNA expression in response to BMP4 treatment is denoted by a *P* value greater than 0.05.

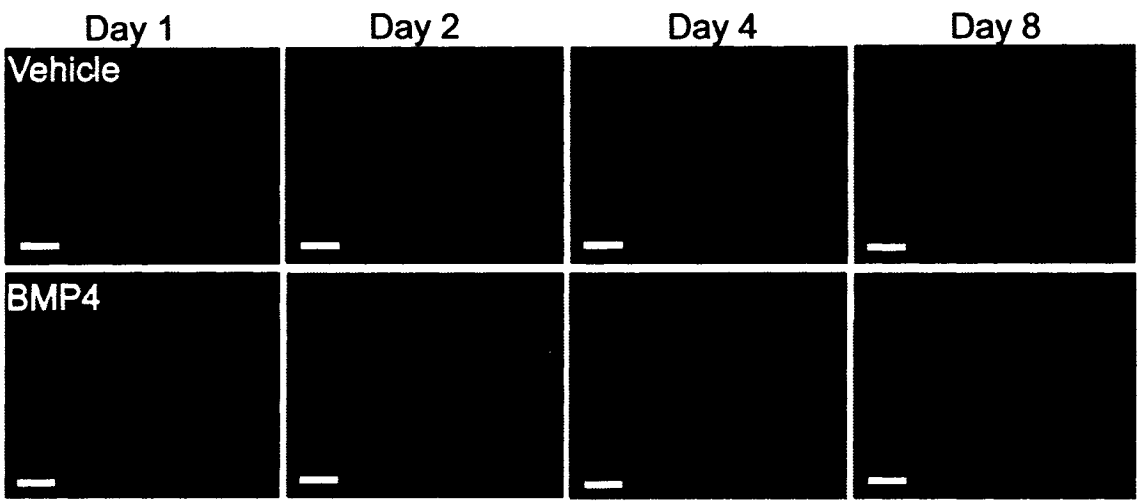


Figure 4-4. BMP4 induces the accumulation of αSMA staining in OvCa cells. Representative OvCa cell sample treated with vehicle (upper panels) or BMP4 (10 ng/mL; bottom panels) for up to 8 days. Media was replenished every 2 days along with treatments. Expression of alpha smooth muscle actin was assessed by immunofluorescence (green) and Hoechst 33258 staining to reveal nuclei (blue). Bar, 50 μm. Similar results were obtained using primary cells from two OvCa samples in triplicate.

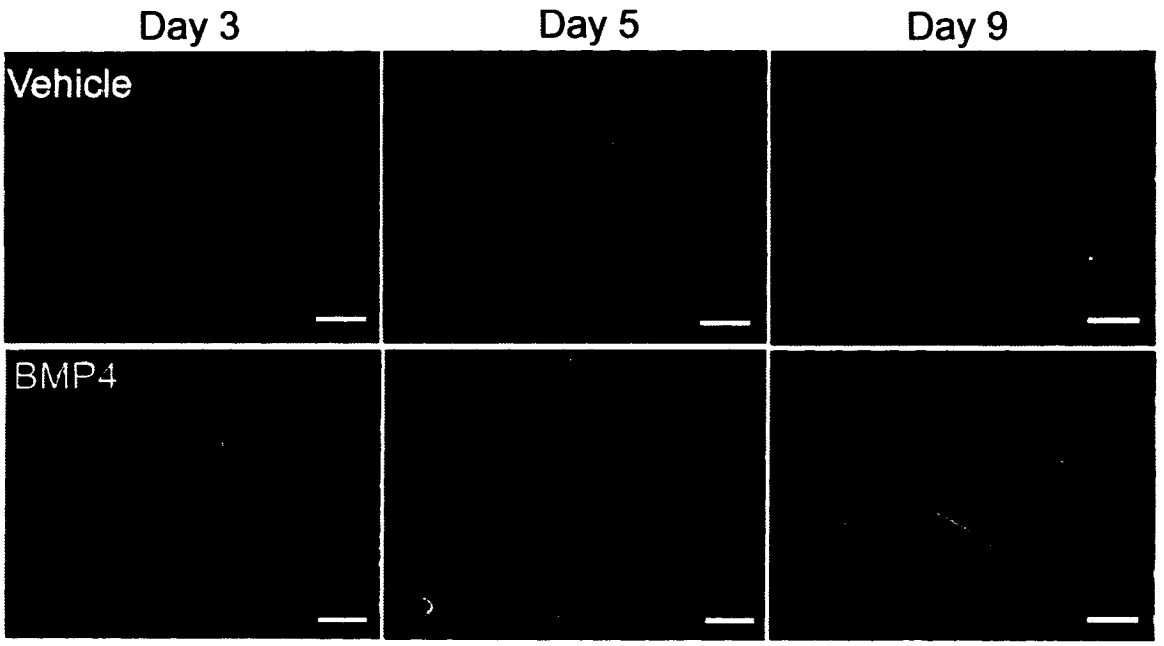


Figure 4-5. BMP4 induces a committed morphological change. OvCa cells were treated with vehicle or BMP4 (10 ng/mL) for 48h, and the BMP4 stimulus was removed by replenishing the culture medium without any BMP4 present. OvCa cells were observed for an additional: 1 day (Day3), 3 days (Day5), and 7 days (Day9) through fluorescence microscopy and digital imagery. OvCa cells were stained with rhodamine-phalloidin (red) to reveal the organization of filamentous stress fibers in response to BMP4 removal. Samples were co-stained with Hoechst 33258 to reveal nuclei (blue). Bar, 50 μm.

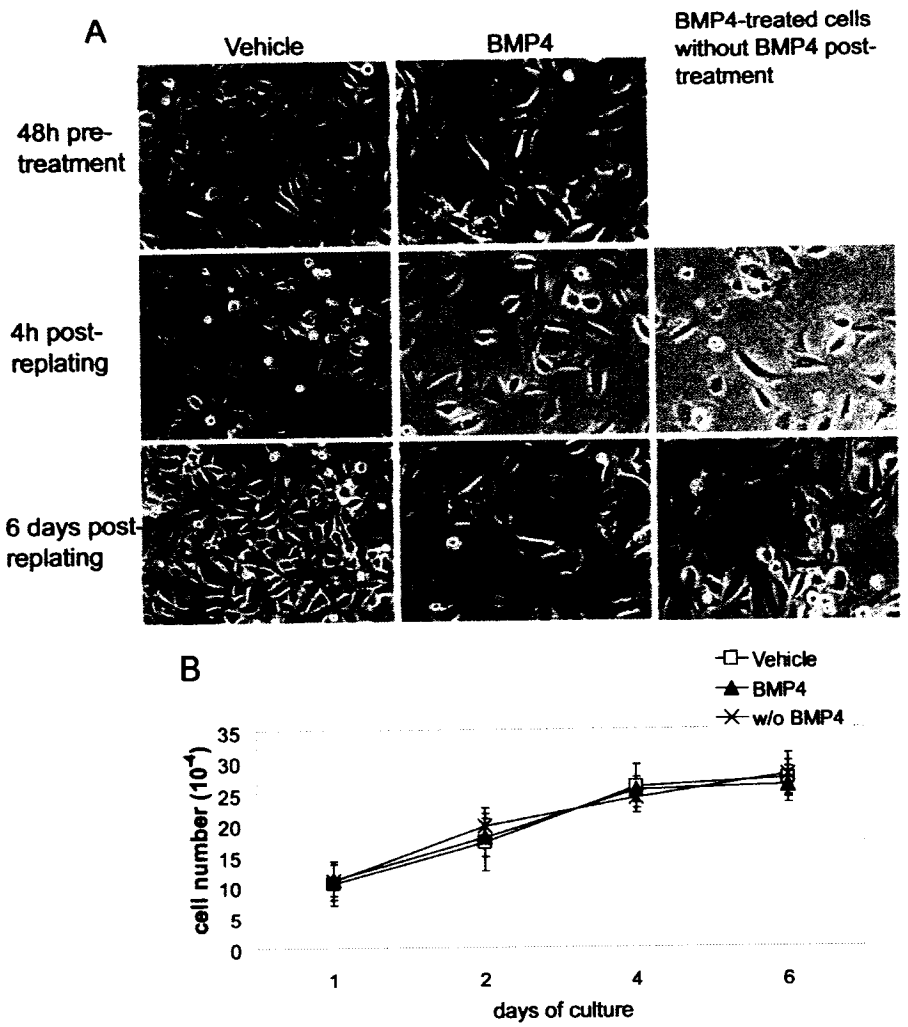


Figure 4-6. OvCa cells treated with BMP4 continue to proliferate following an alteration in cellular morphology. **(A)** Representative images of OvCa cells treated with Vehicle or BMP4 (10 ng/mL) for 48 h were trypsinized and replated in the presence or absence of BMP4. BMP4 treated cells maintained an altered morphology following replating in the presence or absence of BMP4. Cells were visualized by phase contrast microscopy. Magnification 200x. **(B)** The OvCa cells treated in **(A)** were also used to determine rates of cellular proliferation. The graph demonstrates the increase in cell number over days in culture for all treatment groups. BMP4-treated cells received fresh BMP4 (10 ng/mL) every 2 days during the entire culture period. Bars represent standard deviation for data collected using 3 OvCa patient samples in three independent experiments in triplicate.

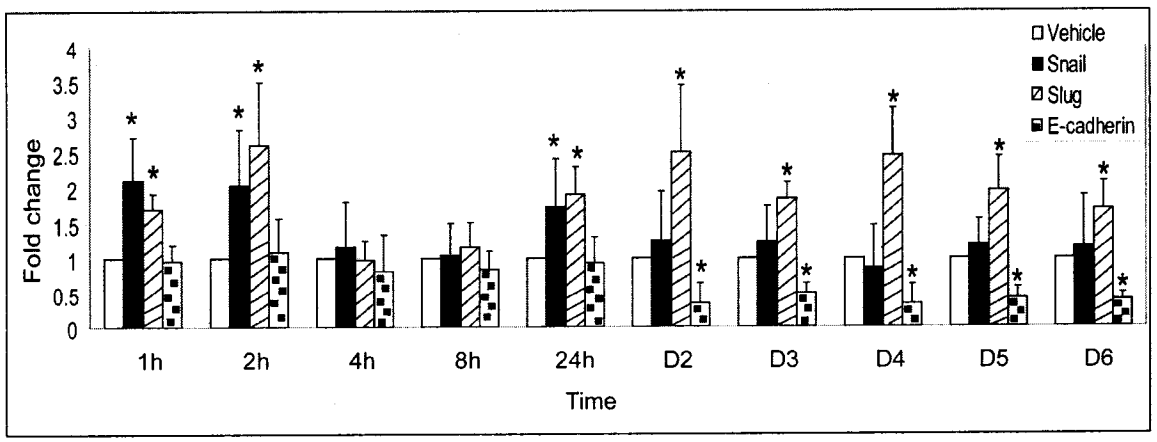


Figure 4-7. BMP4 alters *Snail*, *Slug* and *E-cadherin* mRNA expression in OvCa cells. Mean data from 5 OvCa cell samples showing expression of various EMT markers (Snail, Slug and E-cadherin) assessed using QPCR over a 6 day period in cells treated with vehicle or BMP4 (10 ng/mL). Data are shown as fold change in expression between BMP4-treated relative to vehicle-treated cells. Bars represent standard deviation from three independent experiments conducted in triplicate. $*P < 0.05$.

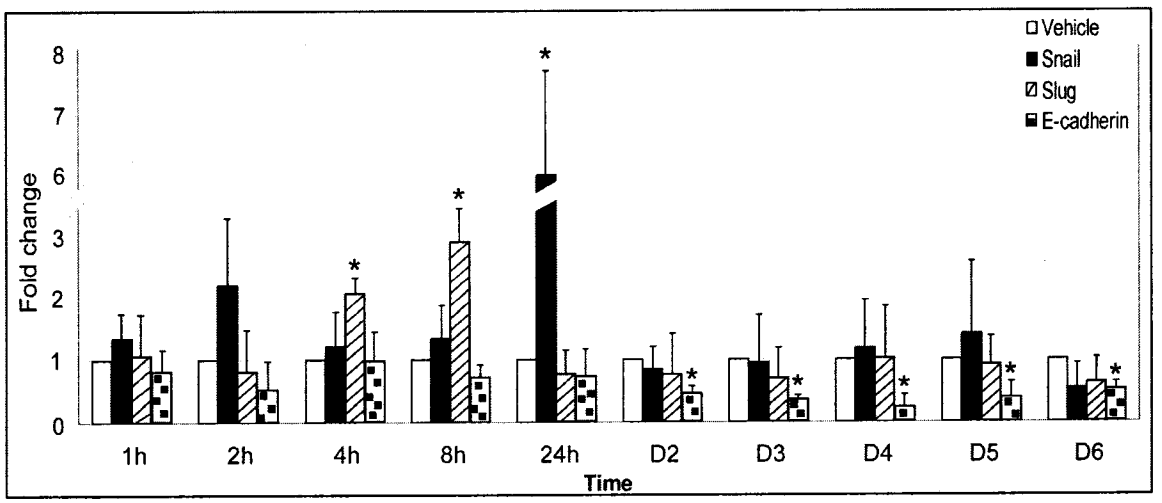
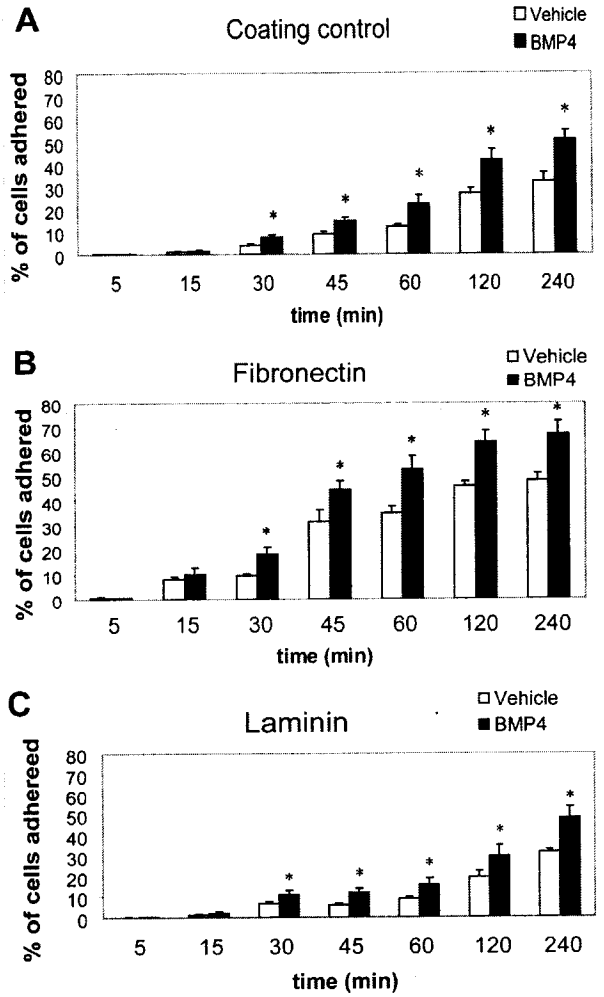


Figure 4-8. BMP4 alters Snail, Slug and E-cadherin protein expression in OvCa cells. Mean data from 3 OvCa cell samples showing expression of Snail, Slug and E-cadherin protein assessed over a 6 day period in cells treated with vehicle or BMP4 (10 ng/mL). Data are shown as fold change in protein expression between BMP4-treated relative to vehicle-treated cells. Bars represent standard deviation of protein expression from three patient samples. $*P < 0.05$.



D	% of cells adhered at 240 min	
	Vehicle	BMP4
coating control	32.1	50.7
collagen I	41.2	58.8
collagen I V	34.3	55.1
laminin	31.5	48.2
fibronectin	48.1	67.2
vitronectin	37.5	53.7

Figure 4-9. OvCa cell reattachement is increased in response to BMP4.
Representative data from one patient sample showing OvCa cells that were pre-treated with BMP4 (10 ng/mL) for 48h, radiolabelled with ^{3}H-amino acids, lifted and re-plated onto: **(A)** coating control (1% BSA in PBS), **(B)** fibronectin or **(C)** laminin. The percentage of cells that adhered was assessed up to 240 min post-plating. **(D)** The percentage of cells that adhered at 240 mins is shown for all ECM substrates tested. Bar represent standard deviation from three independent experiments conducted in triplicate. $*P < 0.05$. Similar results were obtained using primary cells from 3 OvCa samples.

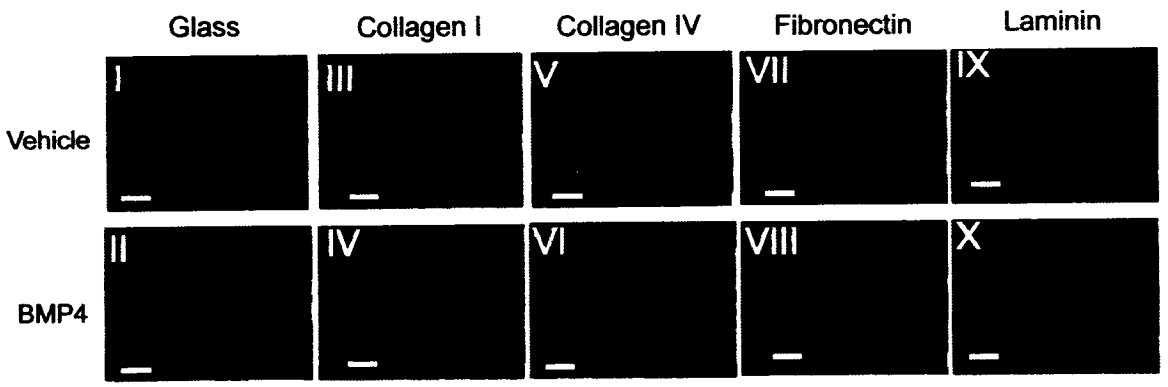


Figure 4-10. OvCa cell morphology on various ECM substrates. Representative images of cell morphology of vehicle (upper panels) or BMP4 (10 ng/mL; lower panels) treated OvCa cells plated on various surfaces for 7 days. (I and II) glass; (III and IV) collagen I; (V and VI) collagen IV; (VII and VIII) fibronectin; (IX and X) laminin. Morphology was visualized by rhodamine-phalloidin staining (red) and fluorescence microscopy. Bar, 50 μm. Similar results were obtained using primary cells from 4 OvCa samples.

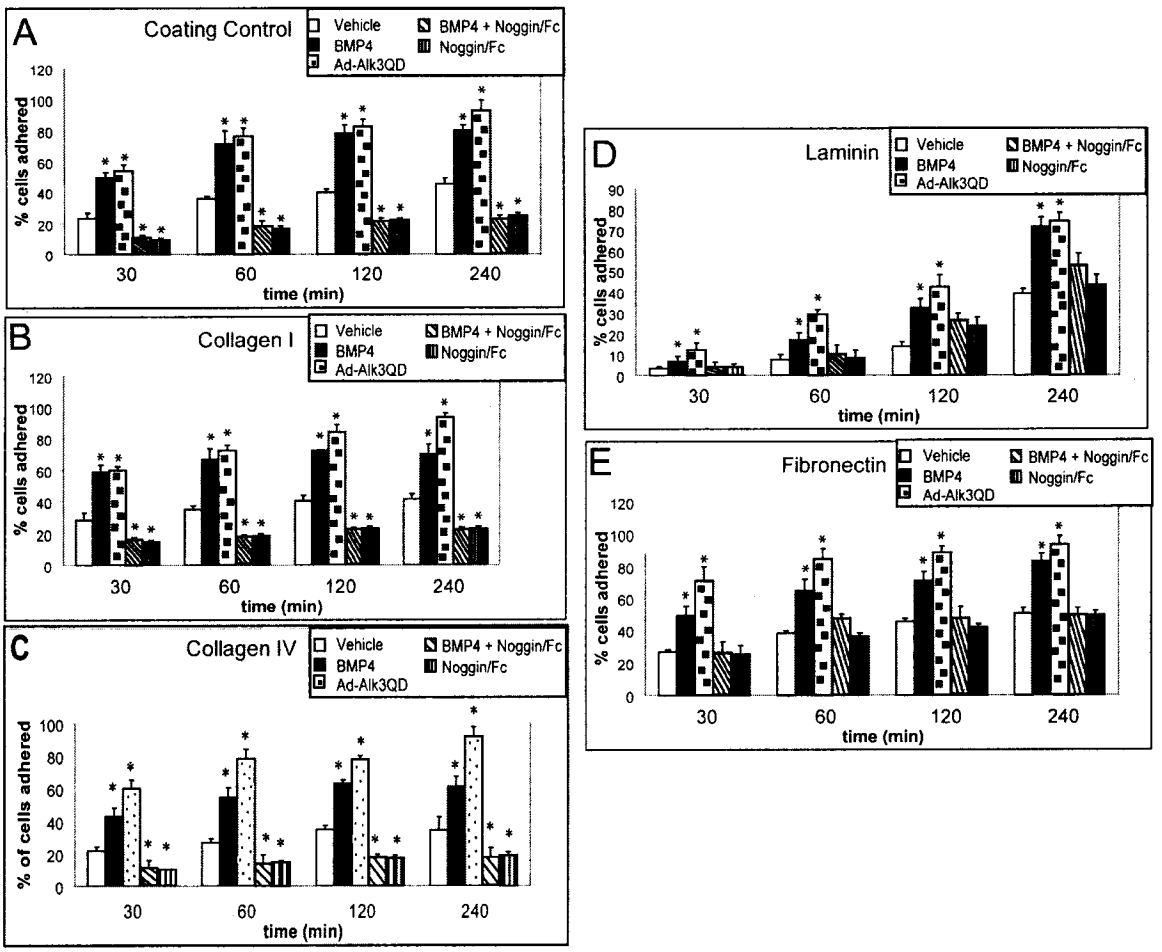


Figure 4-11. Ad-ALK3QD and noggin/Fc modulate OvCa adhesion. Representative data from one patient sample showing OvCa cells that were pre-treated with vehicle, BMP4 (10 ng/mL), Ad-ALK3QD (MOI of 200), BMP4 (10 ng/mL) and Noggin/Fc (100 ng/mL) or Noggin/Fc (100 ng/mL) for 48 h, radiolabeled with ^{3}H-amino acids, lifted and replated onto: **(A)** coating control, **(B)** collagen I, **(C)** collagen IV, **(D)** laminin and **(E)** fibronectin. The percentage of the cells that adhered was assessed up to 240 min post-plating. Bars represent standard deviation from three independent experiments conducted in triplicate. * P <0.05. Similar results were obtained using primary cells from 3 OvCa samples.

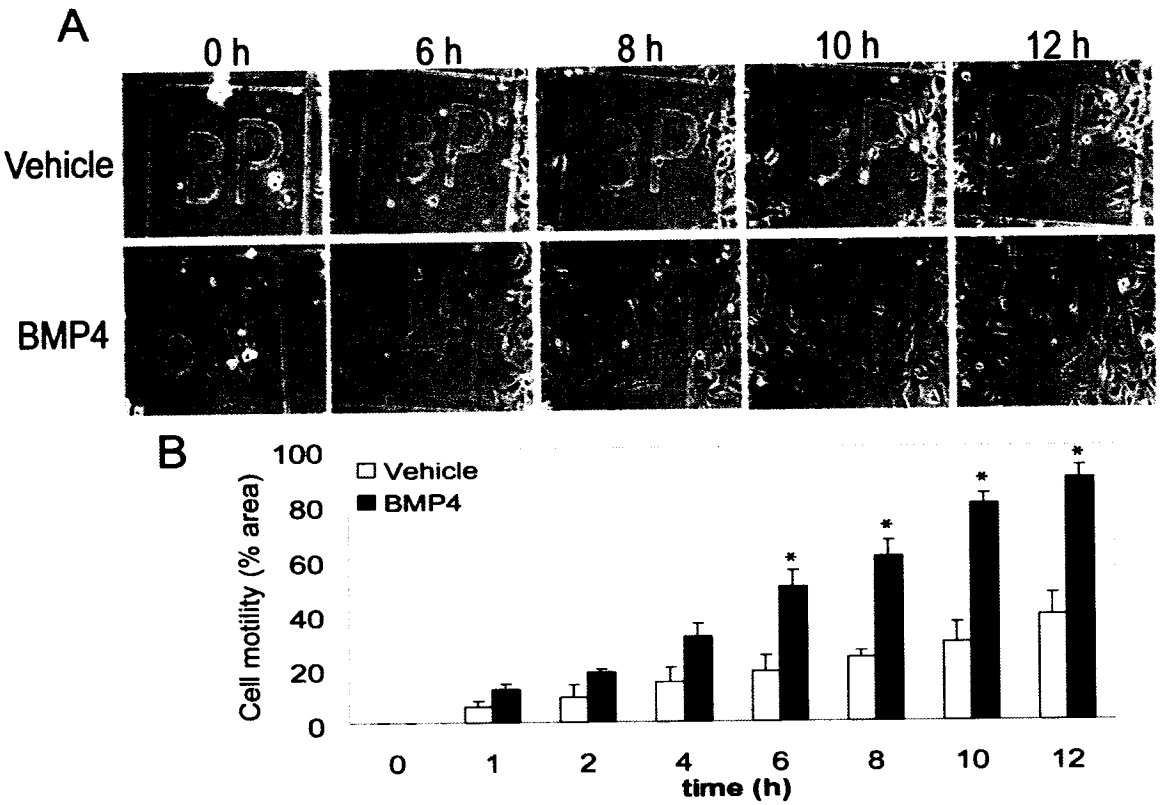


Figure 4-12. BMP4 increases OvCa cell motility. **(A)** OvCa cells were cultured onto etched grid coverslips, pre-treated with vehicle (upper panels) or BMP4 (10 ng/mL) (lower panels) for 48h, and a wounding assay was performed. The wound was monitored for a period of 12h post-wound. Magnification x 100. **(B)** The percentage of the total area covered by OvCa cells was assessed using image analysis software (Image J). The graph depicts data from a representative patient sample. Similar results were obtained using primary cells from 11 OvCa samples. Bars represent standard deviation from three independent experiments conducted in triplicate. * $P < 0.05$.

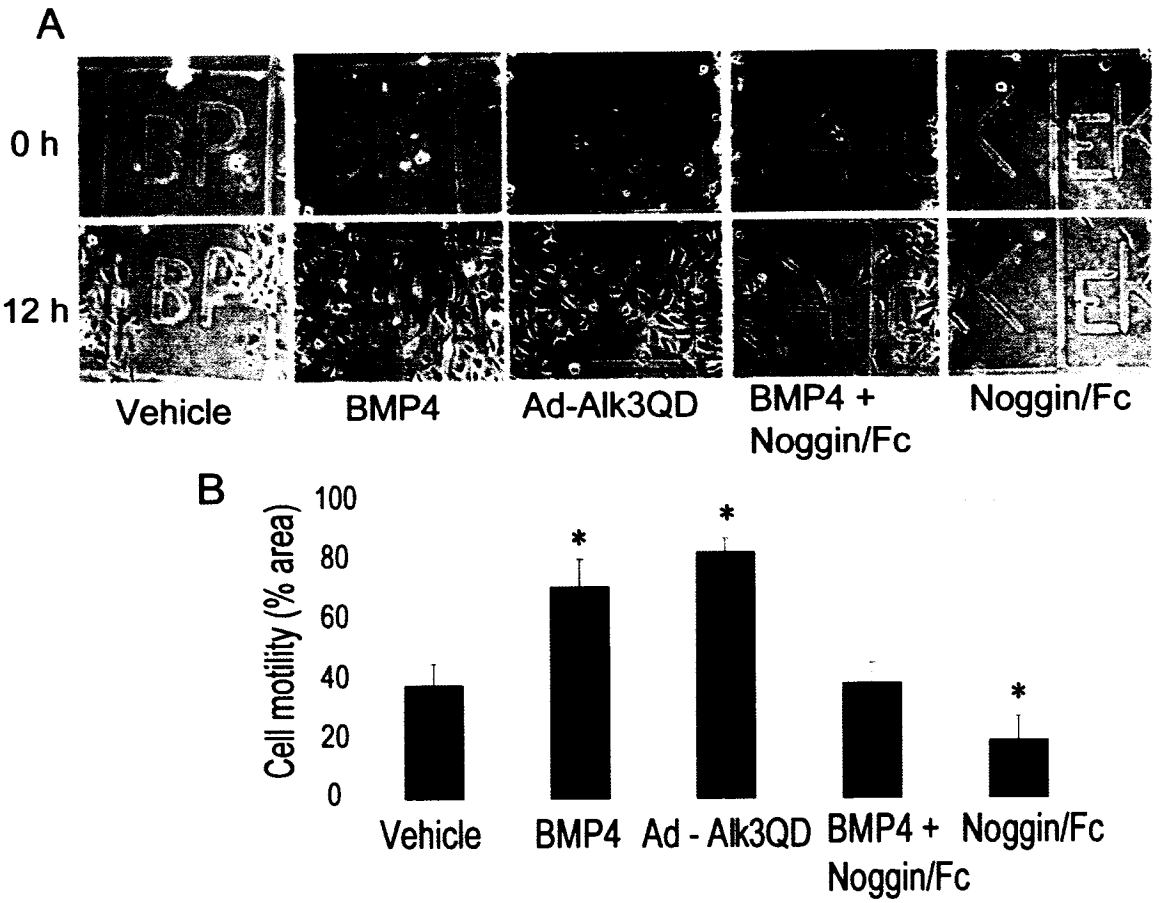


Figure 4-13. Ad-ALK3QD and noggin/Fc modulate OvCa cell motility. **(A)** OvCa cells were cultured on etched grid coverslips, pre-treated with vehicle, BMP4 (10 ng/mL), Ad-ALK3QD (MOI of 200), BMP4 (10 ng/mL) and noggin/Fc (100 ng/mL) or noggin/Fc (100ng/mL) for 48h, and a wounding assay was performed. Images depict the wound space at time 0h (upper panels) and 12h (lower panels). **(B)** The percentage of the total area covered by OvCa cells was assessed using image analysis software (Image J). The graph depicts data from a representative patient sample. Similar results were obtained using primary cells from 3 OvCa samples. Bars represent standard deviation from three independent experiments conducted in triplicate. * $P < 0.05$.

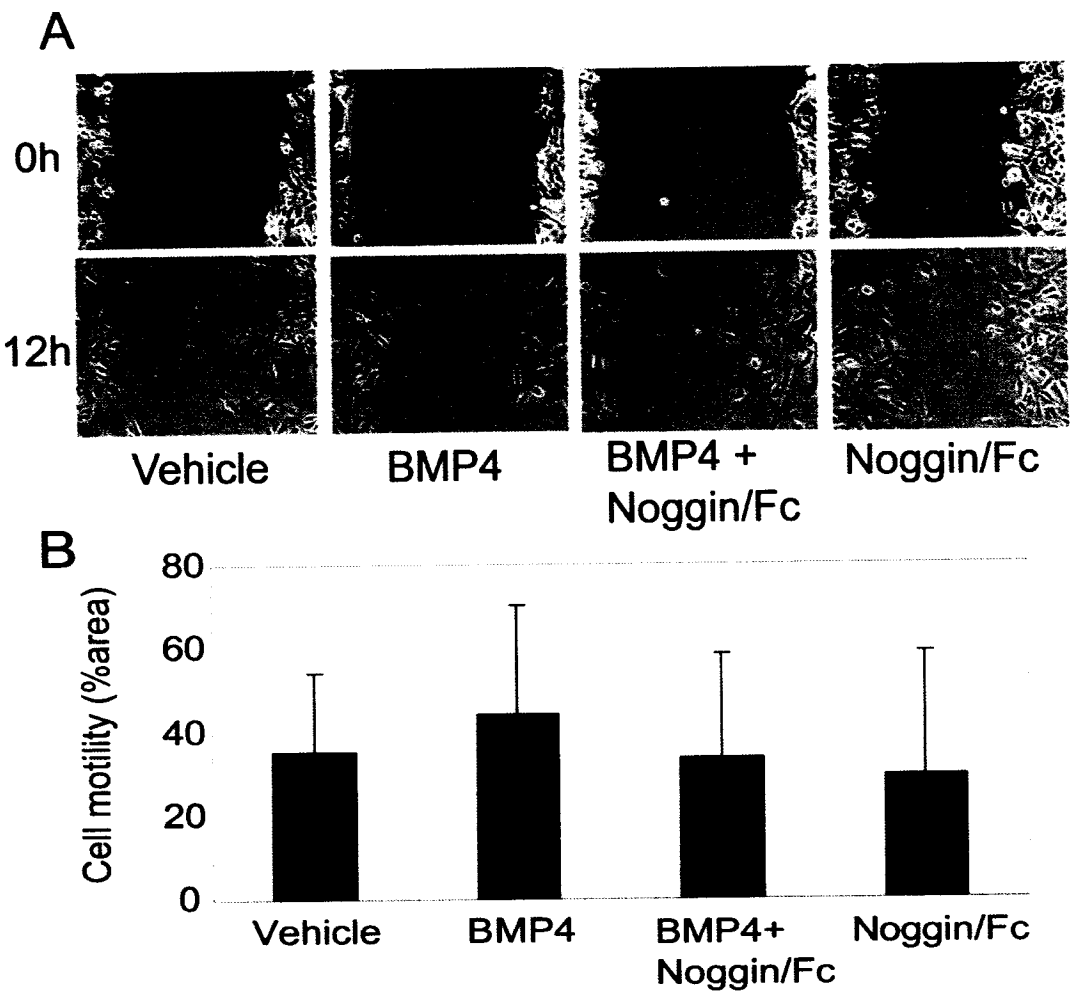
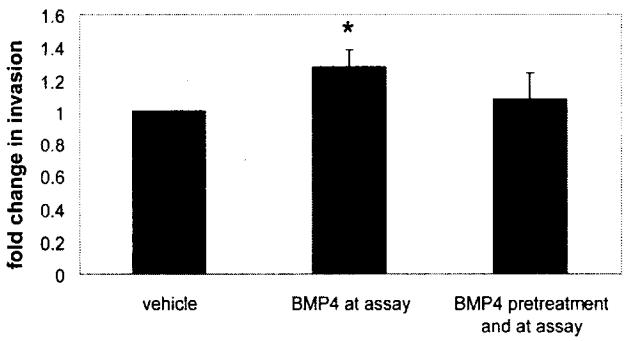


Figure 4-14. BMP4 does not increase normal OSE motility. **(A)** Normal OSE cells were pre-treated with vehicle, BMP4 (10 ng/mL), BMP4 (10 ng/mL) plus noggin/Fc (100 ng/mL) or noggin/Fc (100 ng/mL) alone for 48h, and a wounding assay was performed. Images depict the wound space at time 0h (upper panels) and 12h (lower panels) **(B)** The percentage of the total area covered by OvCa cells was assessed using image analysis software (Image J). The graph represents mean data from 9 normal OSE samples. Bars represent standard deviation from one independent experiment on each of the 9 samples conducted in triplicate.

A

OvCa#	Vehicle	BMP4 at assay	BMP4 pretreatment and at assay	# of replicates
76	59.86	103.29	66.76	9
90	63.11	71.11	73.09	9
93	95	122.58	98.86	9

B



C

OvCa#	NT	Ad-GFP	Ad-Alk3QD	# of replicates
64	7.90	18.28	32.80	9
75	18.67	18.50	59.33	6
85	12.67	30.78	68.89	9
89	3.60	6.23	17.82	18
92	8.67	9.17	17.50	6
101	6.55	8.30	15.62	18

D

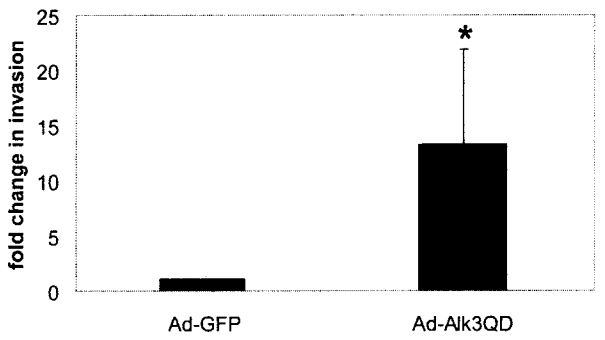


Figure 4-15. BMP4 treatment or ALK3QD expression increases OvCa cell invasion. OvCa cells were pre-treated with vehicle or BMP4 (10 ng/mL) for 48h and at the time of the assay, or naïve cells were treated with BMP4 at the time of the assay and assessed for invasive capabilities through a collagen I matrix. **(A)** The average number of cells that invaded through the collagen I-coated filter is indicated for each treatment. The number of replicate assays for each sample is indicated. Data for 3 OvCa samples (76, 90 and 93) are shown. **(B)** Graphical representation of fold change in invasion relative to vehicle-treated cells that invaded, pooled from all OvCa samples tested, for each treatment. Bars represent standard deviation. $*P < 0.05$. (**C** and **D**) OvCa cells were untransduced (NT) or transduced with Ad-GFP or Ad-ALK3QD. **(C)** The average number of cells that invaded through the collagen I-coated filter is indicated for each treatment. The number of replicate assays for each sample is indicated. Data from 6 OvCa samples (64, 75, 85, 89, 92 and 101) are shown. **(D)** Graphical representation of fold change in invasion relative to Ad-GFP transduced cells that invaded, pooled from all OvCa samples tested, for each treatment. Bar represent standard deviation. $*P < 0.05$.

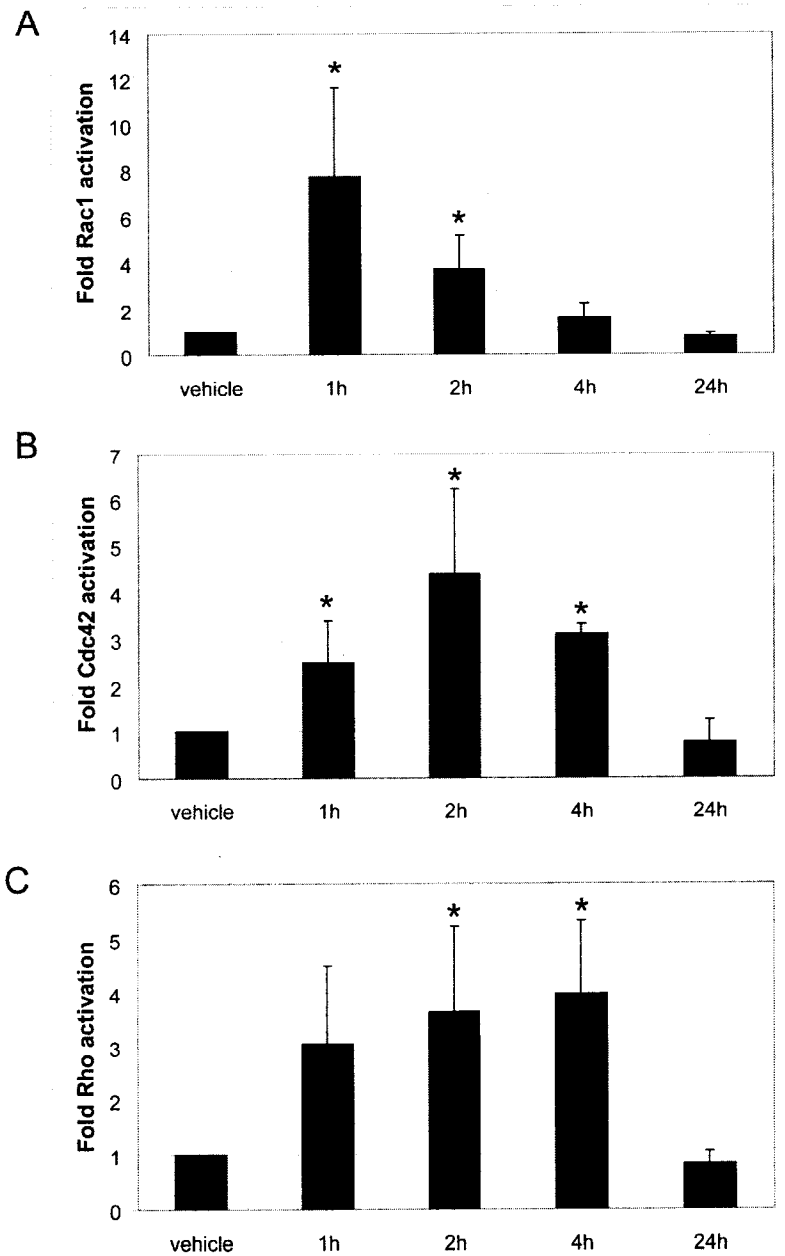


Figure 4-16. BMP4 treatment results in increased Rho-GTPase activation. (A-C) Pooled data from 4 OvCa samples are shown. Cells were treated with vehicle or BMP4 (10 ng/mL) for up to 24h (1, 2, 4 and 24h post-BMP4 addition) and Rho-GTPase activation (Rho, Rac1 or Cdc42) was assessed. Data are expressed as fold change in activation compared with time-matched vehicle-treated samples. Bars represent standard deviation from 3 independent experiments conducted in triplicate. $*P < 0.05$.

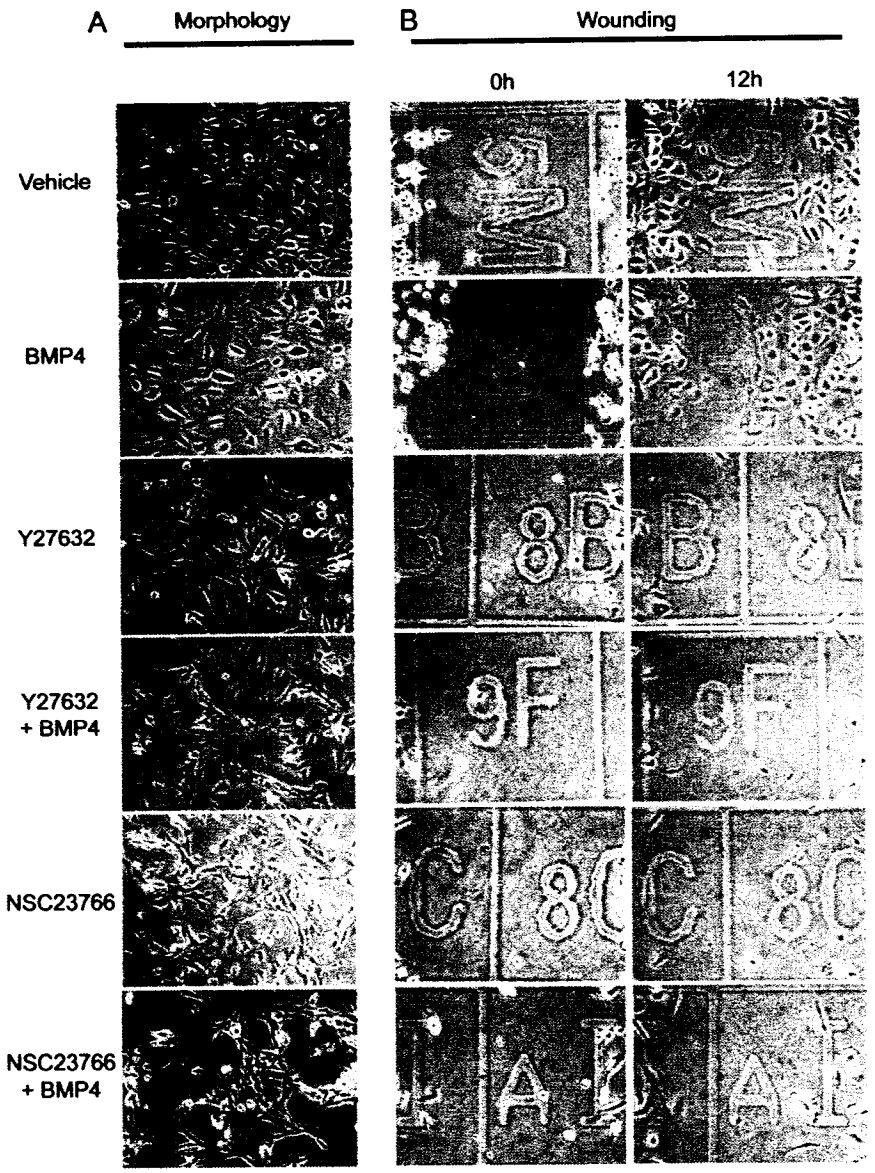


Figure 4-17. Rho GTPase inhibitors significantly change OvCa morphology and decrease OvCa cellular motility. **(A)** OvCa cells were pre-treated with vehicle or BMP4 (10 ng/mL) for 48 h. The night before assessing morphology (approximately 18 h), OvCa cells were treated with either a ROCK inhibitor (Y27632) or a Rac1 inhibitor (NSC23766) in addition to vehicle or BMP4 treatment, and morphology was assessed through phase-contrast microscopy **(B)** OvCa cells were cultured on etched-grid coverslips, and pre-treated for 48 h with vehicle or BMP4 (10 ng/mL). The night before producing the wound, OvCa cells were treated with either a ROCK inhibitor (Y27632) or a Rac1 inhibitor (NSC23766) in addition to vehicle or BMP4 treatment, and a wounding assay was performed the next day where cellular motility was assessed for a 12 h period post-wounding. Images depict the wound space at time 0h (left panels) and 12h (right panels). Similar results were obtained from 2 OvCa patient samples.

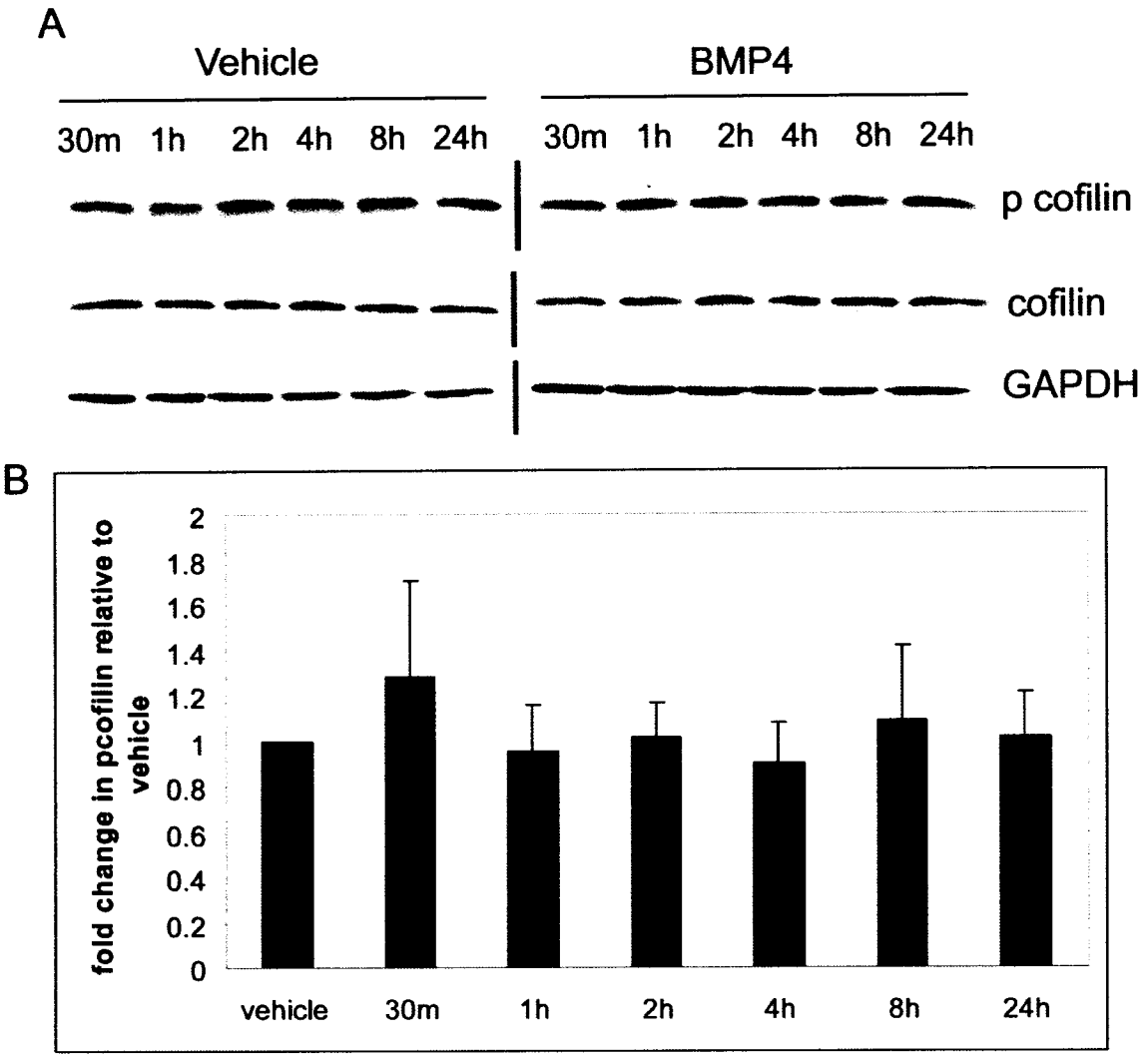


Figure 4-18. BMP4 does not increase cofilin phosphorylation in OvCa cells. (A) Representative western blot of one OvCa patient sample treated with vehicle (left) or BMP4 (10ng/mL; right) for the indicated times. Phospho-cofilin (p cofilin), total cofilin (cofilin) and GAPDH protein levels were subsequently measured using western blots and digital imagery. Black bars delineate separate blots. **(B)** Graphical representation of the fold change in cofilin phosphorylation in response to BMP4 treatment, relative to time-matched vehicle treated cells. Bars represent standard deviation from 3 OvCa patient samples assessed once.

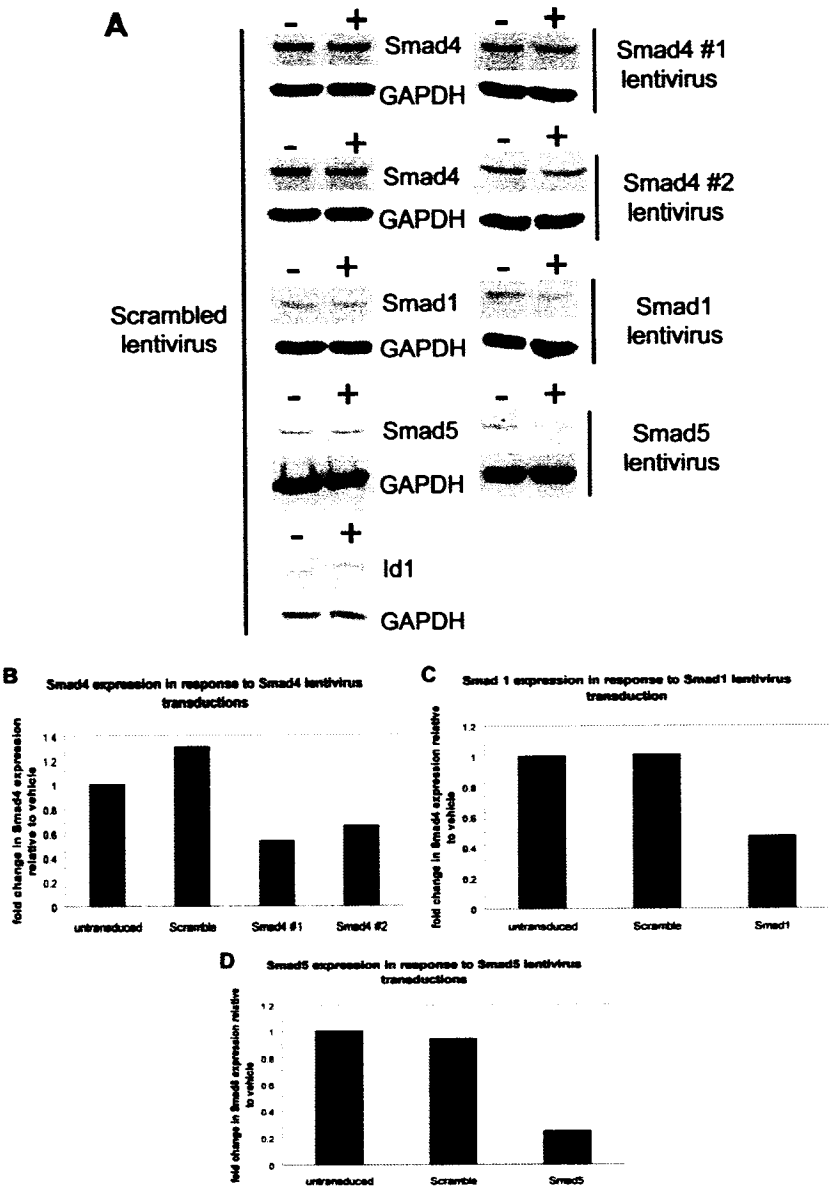


Figure 4-19. Smad lentivirus transduction effectively decreases Smad protein expression in OvCa cells. **(A)** Images of a representative OvCa patient sample that was untransduced (-) or transduced (+) with lentivirus constructs expressing shRNA molecules targeting Smads 1, 4 and 5 mRNA for degradation. Smad protein expression was subsequently assessed through western blotting and digital imagery. A scrambled lentivirus construct with no intended mRNA target was used as a lentiviral expression control to transduce OvCa cells. GAPDH protein expression was used to assess for protein loading. **(B-D)** Graphical representation of the fold change in **(B)** Smad 4 **(C)** Smad 1, and **(D)** Smad 5 protein expression in **(A)** in response to **(B)** Smad 4 #1 and #2, **(C)** Smad 1, and **(D)** Smad 5 lentivirus transduction relative to untransduced Smad expression in OvCa cells. Similar results were obtained using primary cells from 2 OvCa samples.

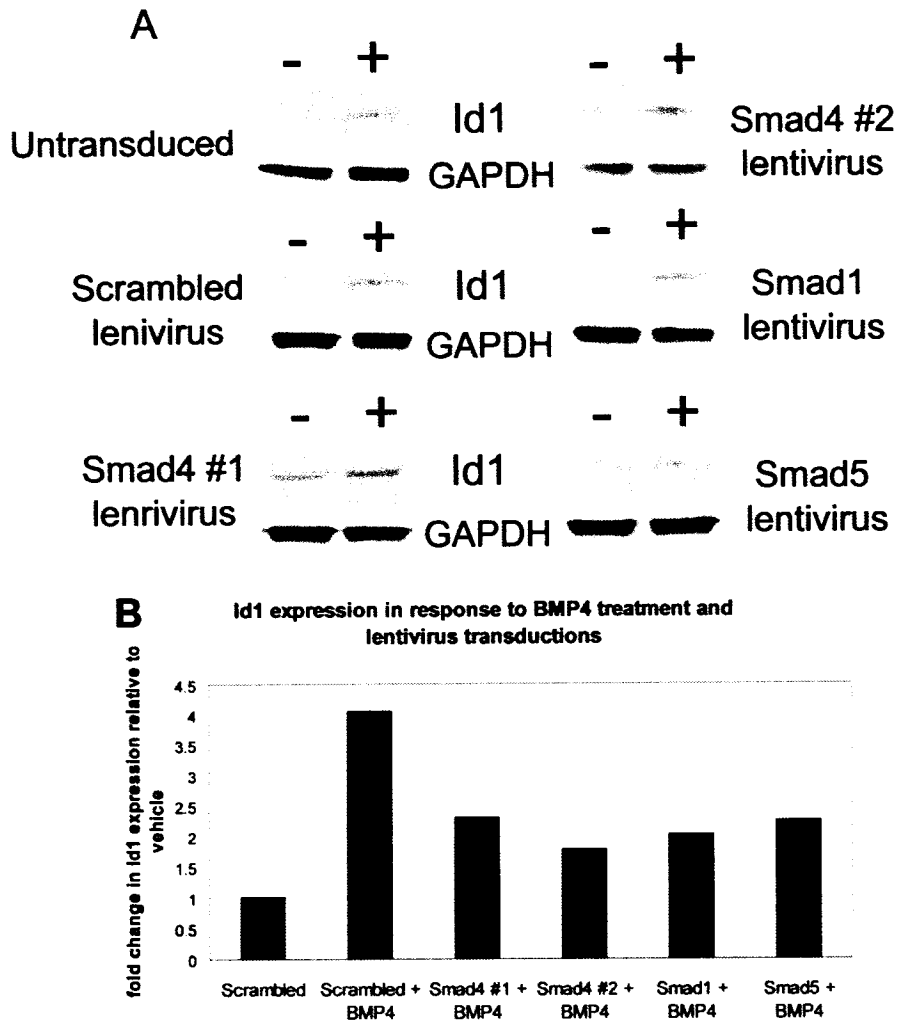


Figure 4-20. Smad lentivirus transduction partially blocks Id1 protein induction in OvCa cells in response to BMP4 treatment. **(A)** Images of a representative OvCa patient sample transduced with lentivirus shRNA constructs targeting Smads 1, 4, and 5 mRNA for degradation. A scrambled lentivirus construct with no intended mRNA target was used as a lentiviral expression control to transduce OvCa cells. OvCa cells were subsequently treated with vehicle (-) or BMP4 (10 ng/mL; +) for 90 min, and Id1 protein induction was assessed through western blotting and digital imagery. GAPDH protein levels were assessed for protein loading. **(B)** Graphical representation of the average fold change in Id1 induction in response to Scrambled, Smad 4#1, Smad 4#2, Smad 1 and Smad 5 lentivirus transduction and BMP4 treatment, relative to lentiviral transduction alone (vehicle). Similar results were obtained using primary cells from one other OvCa sample.

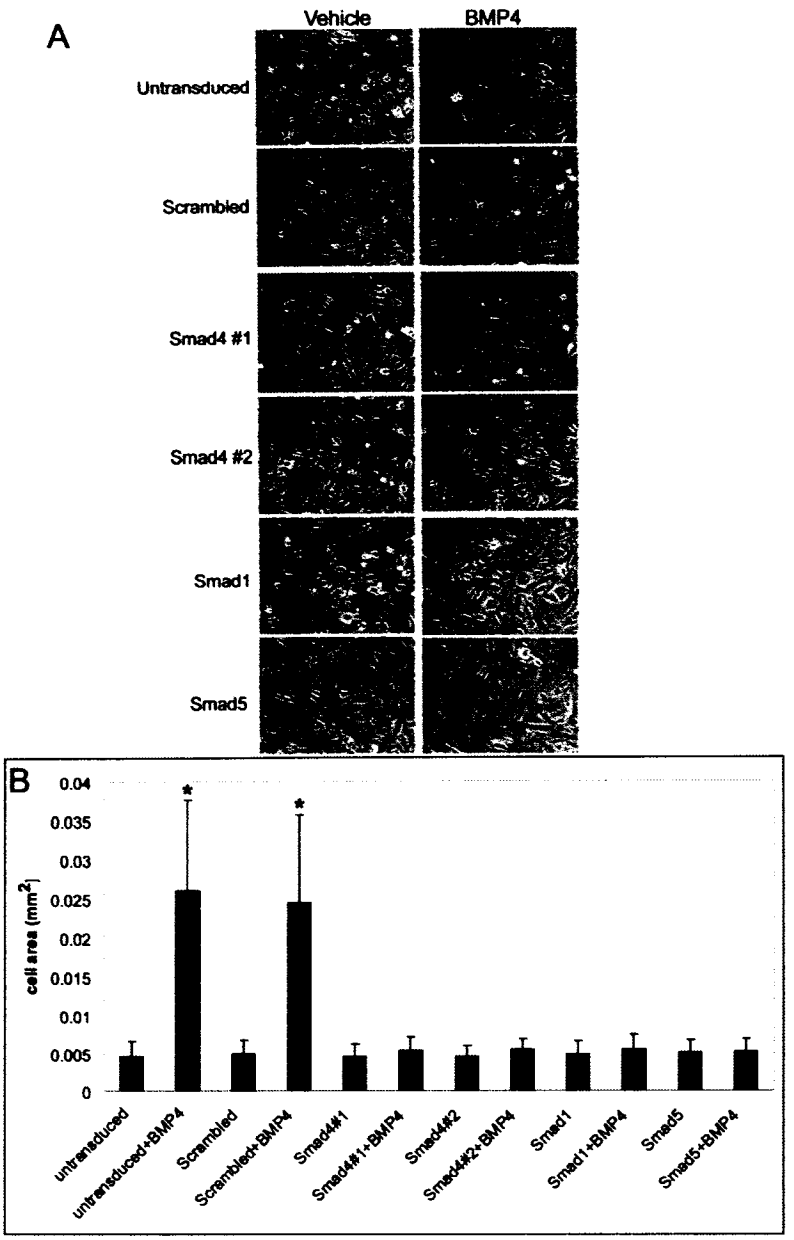


Figure 4-21. Smad lentivirus transduction partially blocks the BMP4-induced phenotypic change in OvCa cells. A) Representative images of OvCa cells transduced with lentivirus constructs targeting Smads 1, 4, and 5 mRNA for degradation, and subsequently treated with vehicle or BMP4 (10 ng/mL) for 4 days. A scrambled lentivirus construct with no intended mRNA target was used as a lentiviral expression control to transduce OvCa cells. Cell morphology was visualized by phase-contrast microscopy and digital imaging. Magnification x 100. Similar results were obtained using primary cells from 3 OvCa samples. **B)** Graphical representation of the average cell area in mm^2 was measured from 3 different patient samples for each treatment. Bars represent standard deviation from three independent experiments conducted in triplicate. * $P < 0.05$.

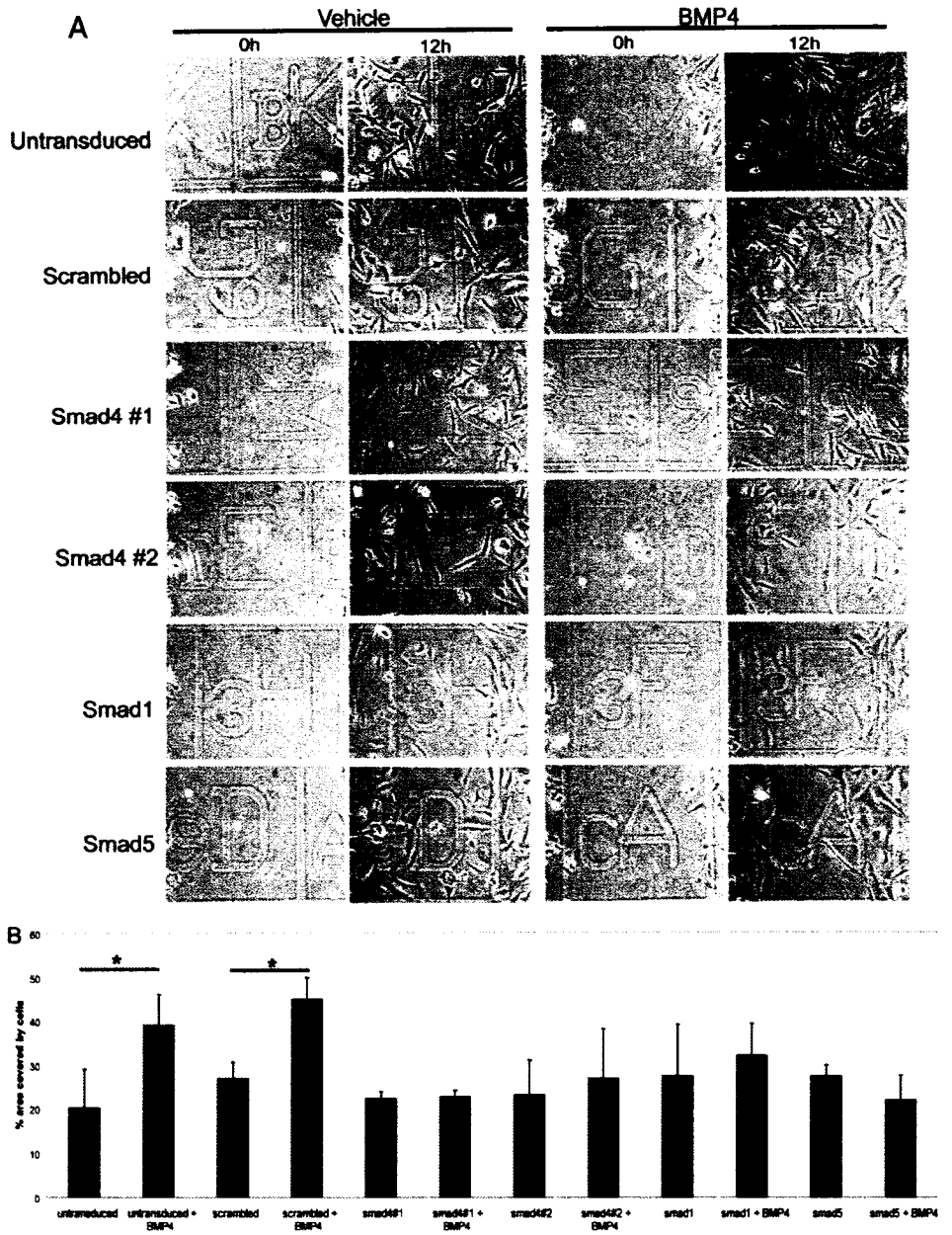


Figure 4-22. Smad lentivirus transduction blocks the BMP4-induced increased in OvCa cell motility. **(A)** Representative images of OvCa cells transduced with lentivirus constructs targeting Smads 1, 4, and 5 mRNA for degradation, and their motility was assessed in response to treatment with vehicle or BMP4 (10 ng/mL) through a wounding assay. A scrambled lentivirus construct with no intended mRNA target was used as a lentiviral expression control to transduce OvCa cells. Images depict the wound space at time 0h and 12h post-wounding. **(B)** The percentage of the total area covered by OvCa cells was assessed using image analysis software (Image J). The graph depicts data from one representative patient sample. Similar results were obtained using primary cells from 3 OvCa samples. Bars represent standard deviation from three independent experiments conducted in triplicate. * $P < 0.05$.

CHAPTER 5 – AUTOCRINE TGFß SUPERFAMILY SIGNALLING DIFFERENTIALLY CONTROLS OVARIAN CANCER CELL BEHAVIOUR

5.1 SUMMARY

TGFß superfamily signalling participates in a variety of normal and pathophysiologic cellular processes. Despite several reports demonstrating the presence of TGFß superfamily signalling pathways in OvCa cell lines and primary cultures, few reports examine the functional outcome of these pathways. Herein we show that both primary human normal OSE and OvCa cells possess intact BMP, TGFß and Activin signalling pathways, where treatment with exogenous ligand will stimulate Smad phosphorylation and target gene upregulation that can be modulated by specific inhibitors. Stimulation of OvCa cells with BMP6, TGFß1 and Activin A ligands induced morphologic changes characterized by cell spreading that was partially blocked by signalling inhibitors. Interestingly, TGFß1 and Activin A induced significant inhibition of OvCa growth and motility, while BMP6 increased cellular motility, having no effect on proliferation. Furthermore, inhibition of autocrine TGFß1 and Activin A signalling resulted in a significant increase in cellular growth and motility to above control levels, whereas inhibition of BMP6 signalling had no effect. Taken together, these results suggest that a balance between BMP and TGFß/Activin signalling is maintained within normal OSE and/or OvCa cells, and that this balance may be altered to favor BMP signalling during OvCa metastatic progression.

5.2 INTRODUCTION

The transforming TGFß superfamily of ligands consists of over 30 different members, exerting a multitude of effects in many adult tissues and playing significant roles in cancer development and progression (Jakowlew, 2006). Many subfamilies of signalling molecules exist within this superfamily including TGFß, BMPs and Activins. BMPs signal through a complex of type I (ALK2, 3, 6) and type II (BMPRII) serine/threonine kinase transmembrane receptors, subsequently activating the intracellular Smads 1, 5, and 8. By contrast, Activins signal through the ALK4 and ALK7 type I receptors and ActRIIA and ActRIIB type II receptors, while TGFßs signal through the ALK1 and ALK5 type I receptors, and type II receptors TßRII; TGFß and Activins subsequently activate the intracellular Smads 2 and 3. Activated Smads 1, 2, 3, 5 and 8 combine with the common Smad 4 and translocate to the nucleus, affecting target gene expression. It is thought that most OvCas arise from the OSE, a monolayer of cells that covers the entire ovarian surface and which is also involved in the cyclical process of ovulation (Auersperg et al., 2001). Many BMP, Activin and TGFß molecules are expressed in the ovary and play important roles in ovulation and the repair processes that follow (Knight and Glister, 2006), suggesting a possible role for these signals in OvCa pathogenesis or progression. Our laboratory has previously shown that both normal OSE and OvCa cells possess autocrine BMP and TGFß signalling pathways, and that these pathways are involved in regulating OvCa cellular behaviours *in vitro* (Dunfield et al., 2002a; Shepherd and Nachtigal, 2003; Theriault et al., 2007). This chapter further extends these observations to show that in addition to BMP4 and TGFß1, other members of the TGFß superfamily of ligands such as BMP6 and Activin A exhibit autocrine

signalling pathways in OvCa cells. We examine the consequences of BMP6, Activin A and TGFß1 signalling induced with exogenous ligands, and show that BMP6 can increase Smad 1/5/8 phosphorylation, target gene expression and cellular motility without affecting cellular proliferation, whereas Activin A and TGFß1 while increasing Smad 2 phosphorylation and target gene expression, are growth-inhibitory and significantly decrease cellular motility. The use of specific signalling inhibitors showed that autocrine Activin and TGFß signalling decrease cellular growth and motility, whereas autocrine BMP signalling had no effect on cellular growth. Additionally, differences were identified between specific BMP4 and BMP6 responses, showing that BMP4 signalling may have a more pronounced impact on controlling cellular behaviour as compared to BMP6 signalling. We show that some of the cellular effects induced by BMP signalling are opposite to the effects induced by Activin and TGFß signalling, uncovering some of the complex actions this family of signalling molecules exerts on OvCa cells. Taken together, our results suggest that a balance is maintained between BMP, and TGFß or Activin signalling pathways and this balance may be altered during OvCa cell progression.

5.3 MATERIALS AND METHODS

OvCa cells were cultured according to section 2.1.1. Cells were treated with recombinant human BMP4, BMP6 and Activin A (R&D Systems) at concentrations ranging from 0.1 ng/mL to 20 ng/mL (3 pM to 550 pM for BMP4 and BMP6; 4 pM to 770 pM for Activin A); recombinant human TGFß1 (Sigma Aldrich Canada) at concentrations ranging from 0.1 ng/mL to 5 ng/mL (4 pM to 200 pM) ; human recombinant noggin/Fc, Chordin and Follistatin (R&D Systems) at concentrations ranging from 25 ng/mL to 200 ng/mL (250 pM to 2 nM for noggin/Fc and Chordin, 625 pM to 5 nM for Follistatin); SB431542 (Sigma Aldrich Canada) at concentrations ranging from 0.25 μM to 2 μM; and LAP (TGFß1 latency associated protein, R&D Systems) at concentrations ranging from 5 ng/mL to 50 ng/mL (7 pM to 715 pM). Noggin was used to inhibit BMP4, Chordin to inhibit BMP6, Follistatin to inhibit Activin, SB431542 to inhibit both Activin and TGFß, and LAP to inhibit TGFß. BMP4, BMP6, Activin A, TGFβ1, noggin/Fc, Chordin, Follistatin and LAP were reconstituted in 0.1% BSA in PBS, while SB431542 was reconstituted in 100% dimethyl sulfoxide (DMSO). Where indicated, either of these diluents was considered as vehicle.

Expression of BMP signalling receptors, Id1, Id3, Snail, Slug and GAPDH was assessed by QPCR using primers specific for each human cDNA sequence (Table 2-1), and performed as in section 2.4.

For wounding assays, OvCa cells were treated for 48 h with either vehicle (DMSO), BMP6 (10 ng/mL; 278 pM), BMP6 (10 ng/mL) + Chordin (100 ng/mL; 1nM), Chordin (100 ng/mL), Activin A (10 ng/mL; 384 pM), Activin A (10 ng/mL) + Follistatin (100 ng/mL; 2.5 nM), Follistatin (100 ng/mL), Activin A (10 ng/mL) +

SB431542 (0.5 μM), TGFß1 (1 ng/mL; 30 pM), TGFß1 (1 ng/mL) + SB431542 (0.5 μM), SB431542 (0.5 μM), TGFß1 (1 ng/mL) + LAP (25 ng/mL; 360 pM), or LAP (25 ng/mL). After the treatment period, a wounding assay was performed as per section 2.5, where media and treatments were replenished after producing the wound.

Growth curves were performed according to section 2.12, where OvCa cells were seeded at low density (2 x 10^4 cells/mL) onto triplicate 6-well culture dishes. The next day (Day 0) cells were counted using a Coulter counter and cells were treated either with vehicle (DMSO), BMP4 (10 ng/mL; 278 pM), BMP4 + noggin/Fc (100 ng/mL; 1 nM), noggin/Fc (100 ng/mL), BMP6 (10 ng/mL), BMP6 (10 ng/mL) + Chordin (100 ng/mL), Chordin (100 ng/mL), Activin A (10 ng/mL), Activin A (10 ng/mL) + Follistatin (100 ng/mL), Follistatin (100 ng/mL), Activin A (10 ng/mL) + SB431542 (0.5 μM), TGFß1 (1 ng/mL), TGFß1 (1 ng/mL) + SB431542 (0.5 μM), SB431542 (0.5 μM), TGFß1 (1 ng/mL) + LAP (25 ng/mL), or LAP (25 ng/mL). Culture medium and treatments were replenished every 2 days, and cell counts were measured every 2 days subsequent to treatment until 6 days post-treatment. Cell counts for each day and treatment were obtained by averaging counts from triplicate wells. Digital cell images at day 6 post-treatment were taken using a Nikon TMS phase-contrast microscope equipped with a Nikon Coolpix 4500 digital camera.

Western blots were performed as per section 2.11. OvCa cells were treated with vehicle, BMP4, BMP6 and Activin A at 0.1, 1, 10 and 20 ng/ml, or TGFß1 at 0.1, 1 and 5 ng/mL, and total protein was isolated at 30, 60, 120 and 240 m post-treatment. Phospho-Smads 1 and 2, total Smads 1, 2, 5 and 8 protein levels were measured in response to treatments as per section 2.11.3. Phospho-Smad 1/5/8, phospho-Smad 2, Smad 1, Smad

2, Smad 5 and Smad 8 protein expression levels were normalized by dividing the protein expression value obtained from the STORM scanner digital analysis program (Image Quant 5.2; Amersham GE Healthcare) for each of the molecules by their respective GAPDH protein expression value. Once these normalized protein values were obtained, the phospho-Smad protein expression in response to ligand treatment was then compared to the vehicle-treated phospho-Smad protein expression and expressed as a fold change in phospho-Smad expression relative to vehicle.

Statistical analyses were conducted according to section 2.14.

5.4 RESULTS

5.4.1 OSE And OvCa Cells Express Components Of TGFß Superfamily Signalling

Previous reports from our laboratory have established that many components involved in transducing TGFß superfamily signals including BMP4, BMP6, BMP2, ALK3, ALK6, BMPRII, TGFß, ALK5, TßRII, Smads 1,2,3 4, 5, and 8 are expressed in normal OSE, OvCa cells and OvCa cell lines (Dunfield et al., 2002a; Shepherd and Nachtigal, 2003). To further delineate whether other components of the BMP, TGFß and Activin families of signalling molecules were expressed in normal OSE or OvCa cells, a QPCR analysis of receptor and ligand expression profiles between normal OSE and OvCa cells was performed on a total of 6 primary human normal OSE and OvCa cells (Figure 5-1). Normal OSE and OvCa cells express, at the mRNA level, the receptors ALK2, ALK3, ALK6 and BMPRII involved in transducing BMP2, BMP4 and BMP6 signals (Ebisawa et al., 1999; Nohno et al., 1995). Additionally, OSE and OvCa cells express ALK1, ALK5, and TßRII, involved in transducing TGFß signals (Lin and Wang, 1992; Shimasaki et al., 2004), as well as ALK4, ALK7, ActRIIA and ActRIIB, involved in transducing Activin and Nodal signals (Attisano et al., 1992). Both normal OSE cells and OvCa cells express the mRNA for secreted molecules involved in extracellular regulation of ligand action, such as BAMBI, Noggin, Chordin, Follistatin and Gremlin (Chen et al., 2004; Keutmann et al., 2004). These results are in agreement with previous reports showing expression of the TGFß superfamily of receptors, ligands and inhibitors in normal OSE, primary OvCa cells and OvCa cell lines (Bristow et al., 1999; Henriksen et al., 1995; Ito et al., 2000; Massague, 1992; Moll et al., 2006; Nilsson and Skinner, 2002; Shepherd and Nachtigal, 2003; Welt et al., 1997; Wong and Leung, 2007). When

expression levels are compared between normal OSE and OvCa cells (Figure 5-1, B), ALK1 expression is increased 3-fold in OvCa cells, ALK7 is increased 7-fold, BAMBI is increased 3-fold, while Follistatin and Gremlin mRNA expression are decreased 6-fold and 3-fold in OvCa cells, respectively. These results confirm that all the components necessary for transduction and regulation of BMP, TGFß and Activin signals are present in both normal OSE and OvCa cells.

5.4.2 TGFß Superfamily Ligands Activate Smad Signal Transduction Pathways In Primary OvCa Cells

Canonical Smad signal transduction pathways encompasses BMP ligands signalling through the specific phosphorylation of Smads 1, 5 and 8, and TGFß and Activin ligands through the specific phosphorylation of Smads 2 and 3 (Waite and Eng, 2003); the activation pattern of Smads in primary human OvCa cells in response to exogenous BMP4, BMP6, TGFβ1 and Activin A remains unknown. In order to clarify this activation pattern, OvCa cells were treated with increasing doses of ligand for up to 4 h. Phospho-Smad (pSmad) 1/5/8, phospho-Smad 2, and total Smad 1, 2, 5 and 8 protein expression levels were determined through western blotting and digital imagery. Figure 5-2 A shows a representative protein expression pattern from one OvCa patient sample after 2 h of treatment. BMP4 and BMP6 increased pSmad 1/5/8 protein levels while TGFß1 and Activin A increased pSmad 2 expression 10- and 5-fold, respectively. Ten ng/mL of BMP4 produced a peak pSmad 1/5/8 phosphorylation at 2 h, while 20 ng/mL of BMP6 produced a similar peak after 4 h (Figure 5-2, B). TGFß1 and Activin A induced peak pSmad 2 levels after 1 h of treatment. Total levels of Smads 1, 2 and 5 remained unchanged throughout the treatment period, while total Smad 8 levels decreased with

increasing doses of each ligand. This may be a result of TGFß superfamily signalling directly enhancing Smad 8 protein degradation, or that intracellular antagonists such as the inhibitory Smads 6/7 or co-repressors Smurf or Ski/SnoN are decreasing Smad 8 gene transcription in response to exogenous ligands (Gazzerro and Minetti, 2007). Indeed, TGFß signalling induces Smad 7 mRNA in primary OvCa cells (Dunfield et al., 2002a). The mechanisms responsible for the changes in Smad 8 protein expression remain to be determined. These results confirm that BMP4 and BMP6 signalling strictly activate the BMP-specific Smads 1, 5, and 8, and that TGFß1 and Activin A activate Smad 2 specifically in OvCa cells.

5.4.3 BMP6, TGFß, And Activin A Regulate Downstream Target Genes In OvCa Cells.

Given that OvCa cells express components for BMP, TGFß and Activin A signalling and activate the canonical Smads in response to ligand stimulation, we investigated whether OvCa cells respond to exogenous BMP6, TGFß and Activin A stimulation in terms of target gene expression. We previously determined that BMP4 signalling induced upregulation of the target genes Id1 and Id3 (Shepherd and Nachtigal, 2003) which are also targets of TGFß and Activin signalling (Kondo et al., 2004; Rotzer et al., 2006). OvCa cells were treated and the mRNA of *Id1*, *Id3*, *Snail* and *Slug* was measured using QPCR (Figure 5-3). Ligand doses were chosen based on the K_D (dissociation constant) of each ligand towards their respective receptors (Donaldson et al., 1999; Koenig et al., 1994; Lin et al., 1995), and BMP4 was included as a positive control (Shepherd and Nachtigal, 2003; Theriault et al., 2007). Only the 2 h post-treatment results are shown, as all ligands tested produced the highest change in mRNA

expression at this time point. As an example, Id1 mRNA expression pattern in response to exogenous TGFß ligands is depicted in Figure 5-4. Similar to BMP4 (Shepherd and Nachtigal, 2003), BMP6 induced significant upregulation of *Id1* and *Id3* mRNA; however, BMP6 did not induce *Id1* and *Id3* upregulation to as great a degree as BMP4. TGFß1 stimulated significant increases in *Id1* and *Id3* mRNA expression levels at all doses tested, while higher doses of Activin A were required to upregulate *Id1* and *Id 3* (Figure 5-3, A and B). Snail and Slug, two transcription factors that are markers of EMT, and that have been shown to be modulated in response to TGFß superfamily signalling (De Craene et al., 2005; Hammerschmidt and Nusslein-Volhard, 1993; Theriault et al., 2007) were also measured in response to ligand treatment (Figure 5-3 C and D). BMP4 and TGFß1 induced a significant upregulation in both genes, while Activin A had no effect on *Slug* mRNA at any of the doses tested. BMP6 signalling did not affect either *Snail* or *Slug* expression. Collectively these results indicated that primary human OvCa cells possess intact BMP4, BMP6, TGFß1 and Activin A signalling pathways.

5.4.4 Inhibitors Of TGFß Superfamily Signalling Modulate OvCa Cell Gene Expression.

To further investigate whether these active signaling pathways may be under the control of intracellular and extracellular signalling inhibitors, OvCa cells were treated with doses of ligands capable of inducing target gene expression, in the presence of increasing doses of known inhibitors. *Id1*, *Id3*, *Snail* and *Slug* mRNA levels were subsequently measured as an indication of signalling activity. As expected, noggin/Fc significantly inhibited the increase of all 4 target genes induced by exogenous BMP4 treatment (Figure 5-5 and 5-6). Chordin inhibited the BMP6-induced increase in *Id1* and

Id3 expression, and Follistatin blocked the Activin A-induced increase in *Id1*, *Id3* and *Snail* (Figure 5-5 and 5-6). LAP blocked the TGFß1-induced increases in all genes tested, while SB431542 blocked both TGFß1 and Activin-induced increases in mRNA. Treatment of OvCa cells with inhibitors alone had no effect on basal mRNA levels. Taken together, these results show that the intact BMP6, TGFß1 and Activin A signalling pathways present in primary human OvCa cells can be modulated with endogenous inhibitors or pharmacologic agents.

5.4.5 Autocrine TGFß Superfamily Signalling Differentially Regulates OvCa Cellular Morphology

Ligands of the TGFß superfamily have been shown to produce changes in cellular morphology (Shepherd and Nachtigal, 2003; Theriault et al., 2007; Zavadil and Bottinger, 2005). We previously showed that primary OvCa cells display a cell spreading phenotype in response to BMP4 (Theriault et al., 2007). To examine the cellular response of primary OvCa cells to BMP6, TGFß1, and Activin A, morphology and cellular density was observed after 6 d of ligand treatment. These experiments were performed in the presence or absence of inhibitors, which allowed us to examine the effect of autocrine signalling activity. Cellular density was determined by counting the number of cell bodies present in a defined area of the culture dish (denoted by the black rectangle in the vehicle image, Figure 5-7) in each of the treatment conditions. As with exogenous BMP4 and noggin/Fc, BMP6 treatment produced a morphologic change characterized by cell spreading and a significant decrease in cellular density that was blocked with Chordin (Figure 5-7). Activin A also produced a cell spreading phenotype and decreased cellular density that was significantly inhibited by Follistatin or SB431542

co-treatment (Figure 5-7). Treatment of OvCa cells with noggin/Fc, Follistatin or SB431542 alone caused the cells to become more densely packed as compared to vehicle (Figure 5-7 B and C summarize the changes in cell density). By contrast, OvCa cells treated with Chordin alone did not show a significant difference in morphology or density from vehicle-treated cells. TGFß1 treatment of OvCa cells produced a significant change in cellular morphology (characterized by a change from a typical cobblestone epithelial morphology to a fibroblast-like morphology with the formation of visible stress fibers) and decreased cell density, which could be partially blocked by co-treatment with either SB431542 or LAP. Moreover, LAP showed a reduced ability to block the TGFß1-induced change in morphology as compared to SB431542. In addition, when OvCa cells were treated with either SB431542 or LAP alone, the cells became more densely packed. These results show that in addition to autocrine BMP4 (Shepherd and Nachtigal, 2003) and TGFß1 (Dunfield et al., 2002a) signalling pathways, primary human OvCa cells also possess autocrine BMP6 and Activin A signaling pathways which influence cellular morphology and cellular saturation density.

5.4.6 Autocrine TGFß Superfamily Signalling Differentially Regulates OvCa Cellular Motility

In addition to modulating cellular morphology and density, TGFß superfamily ligands can also regulate cellular motility in a variety of cell types (Massague et al., 2000). We previously showed that BMP4 increased the motility of primary OvCa cells, and that noggin/Fc blocked exogenous BMP4 effects and reduced autocrine-mediated motility (Theriault et al., 2007). Therefore the influence of ligand or inhibitor treatment on OvCa cell motility was examined using a wounding assay. Pre-treatment of OvCa

cells with 10 ng/mL of BMP6 for 48 h significantly increased the percentage of the total area covered by cells 12 h after producing the wound (Figure 5-8 A and E). Chordin blocked the increase in cellular motility induced by BMP6, although no change in motility was seen when Chordin was administered alone. Activin A significantly decreased OvCa cellular motility to below control levels (vehicle; Figure 5-8, B); this decrease could be rescued by co-treatment with Follistatin. Administration of Follistatin to OvCa cells significantly increased cellular motility over control levels, suggesting that autocrine Activin signalling influences OvCa cell motility. Similar results were obtained with SB431542. TGFß1 pre-treatment also produced a decrease in cellular motility that could be blocked by the addition of SB431542 or LAP. As with Follistatin, either SB431542 or LAP alone produced a significant increase in cellular motility as compared to control levels (Figure 5-8, D and E), further showing that primary OvCa cells also possess an autocrine TGFß signalling pathway that modulates cellular motility.

5.4.7 Autocrine TGFß Superfamily Signalling Differentially Regulates OvCa Cellular Growth

Members of the TGFß superfamily of ligands can regulate proliferation in a variety of normal and cancer cells (Burdette et al., 2005; Dunfield et al., 2002a; Jakowlew, 2006). While we have shown that TGFß blocks proliferation of primary human OvCa (Dunfield et al., 2002a) and BMP4 signalling has no effect on primary human OvCa cell proliferation (Shepherd and Nachtigal, 2003; Theriault et al., 2007), the effects of Activin A or BMP6 signalling on primary human OvCa cell proliferation is currently unknown. We determined that there was no change in cell number in response to BMP6, BMP6 plus Chordin, or Chordin alone (Figure 5-9, A). By contrast, Activin A

treatment of OvCa cells significantly reduced proliferation (Figure 5-9, B). The growth inhibitory effect of Activin A was rescued by co-treatment of cells with Follistatin. Interestingly, treatment of OvCa cells with Follistatin alone significantly increased cellular proliferation above vehicle levels. Similar results were obtained when OvCa cells were either co-treated with Activin A and SB431542 or with SB431542 alone (Figure 5-9 B and C). As expected, TGFß1-induced growth inhibition in OvCa cells could be blocked by co-treating cells with TGFß1 and SB431542, but only partially blocked by co-treating cells with TGFß1 and LAP. When OvCa cells were treated with either SB431542 or LAP alone, proliferation significantly increased above vehicle levels. These results point to the undeniable role of autocrine Activin and TGFß signalling in controlling primary OvCa cell proliferation.

5.5 DISCUSSION

We show that primary OvCa cell morphology, motility and proliferation are influenced by BMP6, TGFß1 and Activin A signalling. Moreover, while treatment with exogenous ligands produce exaggerated cellular responses, OvCa cells possess autocrine BMP, TGFß and Activin signalling pathways that endogenously regulate these behaviours. BMP signalling enhances cellular motility without affecting proliferation, whereas TGFß and Activin reduce motility, which is coincident with decreased proliferation. Although TGFß1 and Activin A produce similar gross cellular responses such as morphological changes, decreased motility and reduced proliferation, measurable differences induced by these ligands are apparent in the differential gene expression profile. Our data suggest that cellular activities such as growth and motility are balanced between autocrine BMP4 (Theriault et al., 2007) signalling, and to a lesser extent BMP6, and TGFß or Activin A signalling, highlighting the importance of the tumor microenvironment to influence cell behaviour.

A survey of the mRNA expression of TGFß superfamily receptors and inhibitors in normal OSE and OvCa cells revealed that all components necessary for BMP, TGFß and Activin signalling are expressed. Moreover, all of these signalling pathways are functional in primary OvCa cells. However, differences in receptor and inhibitor mRNA expression levels exist between normal OSE and OvCa cells. ALK1 mRNA is significantly increased in OvCa cells, suggesting that TGFß signalling may be enhanced in OvCa cells as compared to normal OSE cells. Others have shown that TGFß receptors can be highly expressed in late-stage cancers as compared to normal tissues (Roberts and Wakefield, 2003; Safina et al., 2007), and that high ALK1 expression correlates with an

enhanced growth-inhibitory effect in cancer cells (Ungefroren et al., 2007). Furthermore, we show that autocrine TGFß signalling induces growth inhibition in our OvCa cell cultures, indicating that autocrine TGFß may exert these effects not only through ALK5, but also through ALK1. The mRNA for the Nodal type I receptor ALK7 is also overexpressed in our primary OvCa cells, indicating that Nodal signalling may also be enhanced in our OvCa cultures. Indeed, Xu *et al* have shown that Nodal induces apoptosis and inhibition of proliferation in OvCa cell lines through overexpression of the ALK7 receptor (Xu et al., 2004; Xu et al., 2006). We show that the mRNA expression for the TGFß superfamily type I pseudo receptor BAMBI is not only very high in both normal OSE and OvCa cells, but that its expression is significantly increased in OvCa cells as compared to normal OSE cells. This result is in contrast to our functional data showing that BMP, TGFß and Activin signalling pathways are intact and modulate OvCa cell behaviour, indicating that this pseudo receptor may not be crucial in regulating the TGFß superfamily-induced changes in OvCa cellular behaviour. Analysis of the BAMBI protein would provide great insight into the role that it may play in differential regulation of the TGFß superfamily in OvCa cells; our attempts to examine BAMBI protein expression using commercially available antibodies have thus far been unsuccessful. An interesting finding is that the mRNA expression of the BMP inhibitor Chordin in primary OvCa cells remained unchanged when compared with normal OSE cells. These results are in contrast with those by Moll *et al* showing decreased Chordin expression in OvCa cell lines (Moll et al., 2006) that contributes to decreased OvCa cell line motility. In our primary OvCa cells, although Chordin was able to block the BMP6-induced increase in cellular motility, Chordin alone did not alter OvCa cellular motility. These results further

highlight the differences in signalling existing between primary OvCa cells versus cell lines, and how these differences may alter the control of OvCa cellular behaviours *in vitro*.

Follistatin and Gremlin mRNA levels are significantly decreased in OvCa cells compared to normal OSE cells, suggesting that the reduced expression of these inhibitors may contribute to the alteration in the signalling activity of Activin or BMP in OvCa cells. Through the use of inhibitors, we show that autocrine BMP and Activin signalling have functional consequences on OvCa cells in modulating cell growth, morphology and motility, indicating that enhanced BMP and Activin signalling may contribute to the control of OvCa cell functions. However, the physiological consequences of these differential gene expression profiles remain to be determined and will require the quantification of protein levels for each of the respective mRNA signals. These results raise the possibility that TGFß, BMP and Activin signalling in OvCa cells differentially controls OvCa cellular functions and tumor progression.

Our results show that there are marked differences in how BMP4 and BMP6 affect intracellular signalling and cellular behaviours. As with BMP4 treatment, BMP6 showed no effect on cellular proliferation, but significantly increased cellular motility above vehicle-treated levels, and this motility could be blocked by Chordin. However, BMP6 stimulation did not induce *Id1* and *Id3* target gene expression to the same level as with BMP4 stimulation. Peak phosphorylation of pSmad 1/5/8 was achieved with a lower dose of BMP4 as compared to BMP6. Furthermore, Chordin administration alone did not increase cellular density as was seen with noggin/Fc, Follistatin, SB431542, or LAP, or decrease cellular motility to below vehicle levels as was seen with the BMP4

inhibitor noggin/Fc (Theriault et al., 2007). These findings demonstrate that exogenous BMP6 can alter cellular behaviours, however autocrine BMP6 signalling may not play a significant role regulating these OvCa cell responses in contrast to the important autocrine influence of BMP4 (Theriault et al., 2007). Uncovering a role for BMP6 in OSE and OvCa cells remains to be determined.

Both Activin A and TGFß1 signalling show similar effects on OvCa cells. Activin A and TGFß induced peak pSmad 2 phosphorylation with a similar time course, and increased *Id1* and *Id3* gene expression to similar levels. Activin A and TGFß1 also increased *Snail* expression; however while TGFß1 increased *Slug* expression, Activin A did not affect *Slug* mRNA levels. TGFß and Activin A altered cell morphology, but in a manner different from BMP4 or 6 stimulation. TGFß1 and Activin A induced a greater cell spreading effect, as evidenced by a larger cell size and lower cell density as compared to the BMP-induced spreading effect. This may be partly due to the increase in *Snail* mRNA that was seen in response to TGFß1 and Activin A treatment. Snail and Slug are transcription factors involved in the remodeling of epithelial cells into cells with a mesenchymal phenotype. This transformation is characterized by phenotypic changes such as cytoskeletal remodeling into stress fibers, increased cellular flattening and the acquisition of a fibroblast-like morphology (Savagner, 2001). These data suggest that in addition to inducing growth inhibition and decreased motility, TGFß1 and Activin A can also induce a cellular response resembling an EMT through the upregulation of Snail; however this response may not require the simultaneous upregulation of Slug (Cano et al., 2000). By contrast, we have previously shown that BMP signalling also induces an EMT response, but with a concurrent increase in motility (Theriault et al., 2007). These

differential cellular effects exhibited by exogenous TGFß and Activin signalling may underscore the potential dualistic role of these signalling molecules in OvCa cells, which has been previously shown to occur in many other epithelial cancers (Pardali and Moustakas, 2007). As with BMP signalling, the change in morphology induced by TGFß1 or Activin A treatment was blocked by inhibitor administration. However, co-administration of TGFß1 and LAP only partially blocked the TGFß1-induced morphologic change, as compared to co-administration of TGFß1 and SB431542. This result likely reflects the mechanism of action of LAP, an extracellular inhibitor that prevents TGFß1 from interacting with its receptors. By contrast, SB431542 blocks ALK5 as well as ALK4 activation, and thus will block autocrine Activin signalling. This result is consistent with the finding that treatment of OvCa cells with SB431542 produces a marginally higher degree of cell density compared to treatment of OvCa cells with either Follistatin or LAP alone. These results indicate that both autocrine TGFß and Activin signalling contribute to the basal morphology of OvCa cells.

TGFß1 and Activin A showed functionally different effects from BMP signalling on OvCa cell motility and proliferation. TGFß1 and Activin A induced growth inhibition and significantly decreased cellular motility. More importantly, motility and proliferation were significantly increased above control levels in response to inhibitors, showing that autocrine TGFß and Activin signalling inhibits cell motility and proliferation in OvCa cells. Our results indicate that autocrine BMP signalling, and TGFß or Activin signalling differentially modulate OvCa cell behaviours, with the ultimate result of producing opposite proliferation and motility behaviours in these cells.

In the ovary, the OSE is involved in ovulation and the repair processes that follow (Auersperg et al., 2001). Controlled expression of TGFß superfamily ligands at the appropriate times during these processes ensures a proper re-establishment of the integrity of the OSE after each ovulatory cycle (Nilsson and Skinner, 2002). BMP4 has been shown to be produced locally in OSE cells surrounding the ovulation site (Erickson and Shimasaki, 2003), suggesting that BMP4 may participate in OSE repair through promoting cellular division or migration. By contrast, it is thought that TGFß through its growth inhibitory effects on the OSE will prevent the over-proliferation of cells during the ovulatory cycle (Berchuck et al., 1992; Havrilesky et al., 1995; Wong and Leung, 2007), and that Activin signalling will control OSE cellular proliferation through apoptosis (Choi et al., 2001). As with normal OSE, we suggest a balance is maintained between autocrine BMP signalling and TGFß or Activin A signalling in OvCa cells, and that the environmental context and inherent molecular influences to which these cells are exposed will alter this signalling balance and influence the resulting phenotype. We have shown that both TGFß1 (2.3 ng/mL, N=11) and BMP4 (18 pg/mL, N=30) are present in the ascitic fluid from which our primary OvCa cells are derived, indicating that OvCa cells could potentially respond to both of these stimuli during OvCa progression ((Dunfield and Nachtigal, 2003) and unpublished observations). Although TGFß and Activin signalling may be enhanced in our OvCa cells, we have previously determined that BMP signalling is enhanced in OvCa cells versus normal OSE cells (Shepherd and Nachtigal, 2003), and that BMP4 also confers increased motility and adhesive capabilities to OvCa cells (Theriault et al., 2007). Activation of the autocrine BMP4 pathway in OvCa cells of a primary tumor may cause OvCa cell dissemination and

attachment to extra-ovarian sites within the peritoneal cavity, contributing the metastatic progression of the cancer. By contrast, autocrine TGFß or Activin signalling may contribute to reducing tumor growth and spread through the inhibition of cellular growth and motility. The ultimate impact of environmental influences present at the primary tumor site or in the peritoneal fluid on BMP, TGFß and Activin signalling balance remains to be determined. Nevertheless, the fact that in our hands, autocrine BMP signalling induces more aggressive OvCa cellular behaviours suggests that it has a role to play in OvCa cell progression. Thus developing strategies to either block BMP signalling or enhance TGFß and Activin signalling may prove useful in preventing or halting OvCa tumor growth or metastatic progression.

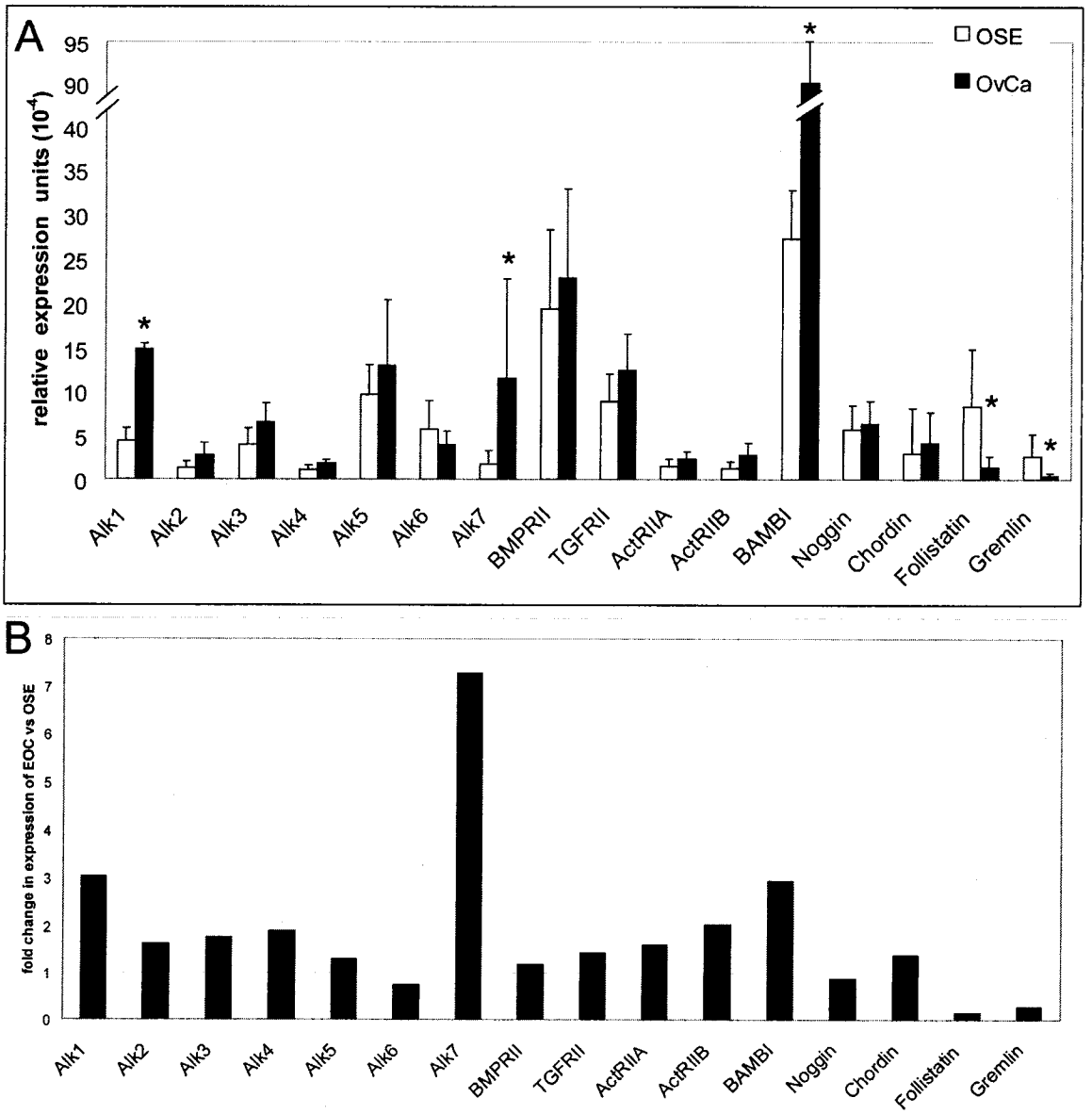
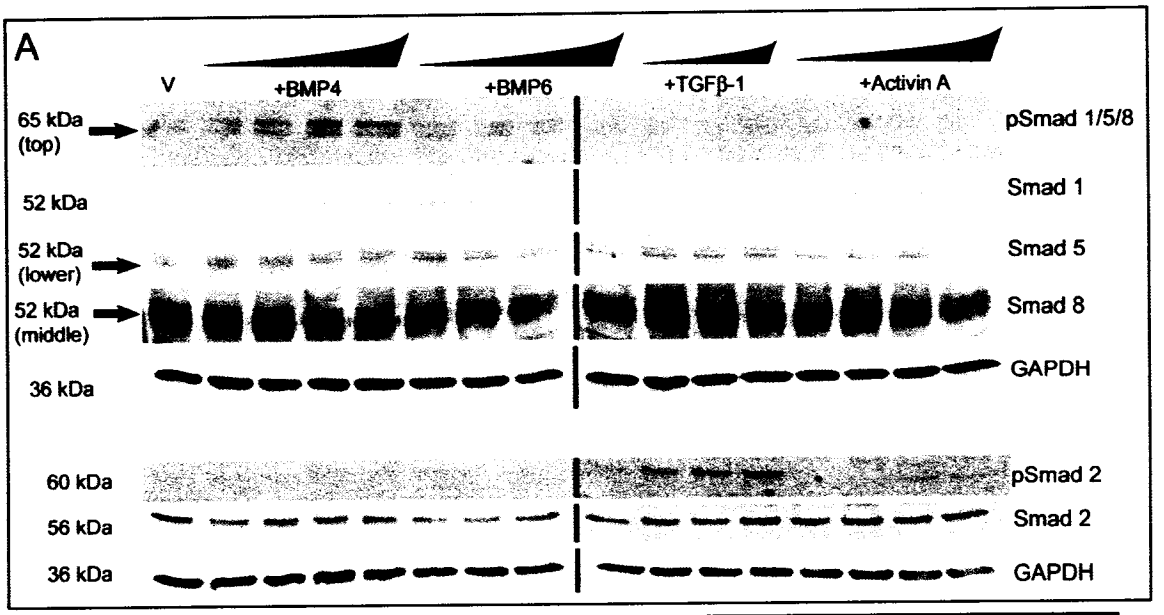


Figure 5-1. OSE and OvCa cells express components of TGFβ superfamily signalling. **(A)** Mean data from 6 normal OSE (white) and primary OvCa samples (black) showing expression of various receptors and inhibitors of the TGFß superfamily of molecules assessed using QPCR. Data is shown as relative mRNA levels of both normal OSE and OvCa mRNA normalized to their respective GAPDH levels, in relative expression units. Bars represent the standard deviation from three independent experiments conducted in duplicate. *$P<0.05$. **(B)** Mean relative expression data from **(A)** expressed as a fold change in mRNA expression of OvCa samples relative to normal OSE samples.



B

Peak Smad Phosphorylation						
	pSmad 1/5/8			pSmad 2		
Ligand	Time	Dose (ng/mL)	Fold change	Time	Dose (ng/mL)	Fold change
BMP4	2h	10	5.7	-	-	-
BMP6	4h	20	5.2	-	-	-
Activin A	-	-	-	1h	10	5
TGFβ-1	-	-	-	1h	1 and 5	10

Figure 5-2. TGFβ superfamily ligands activate Smad signal transduction pathways in OvCa cells. **(A)** Western blots representing expression levels of phospho Smad 1/5/8 (pSmad 1/5/8, 65 kDa, top protein), total Smads 1 (52 kDa), 5 (52 kDa, lower protein), 8 (52 kDa, middle protein), phospho Smad2 (pSmad2, 60 kDa), total Smad 2 (56 kDa) and GAPDH (36 kDa) from a representative primary human OvCa patient sample treated with increasing doses of BMP4, BMP6 and Activin A ranging from 0.1 ng/mL to 20 ng/mL, and TGFβ-1 ranging from 0.1 ng/mL to 5 ng/mL for a period of 2 hrs. V, Vehicle (0.1% BSA/PBS). **(B)** Table representing the dose and time at which each ligand produced peak activation in pSmad protein phosphorylation. The fold change in pSmad1/5/8 or pSmad2 expression was calculated by comparing ligand-treated pSmad expression to vehicle-treated pSmad expression. Data represents mean protein expression data from 3 patient samples.

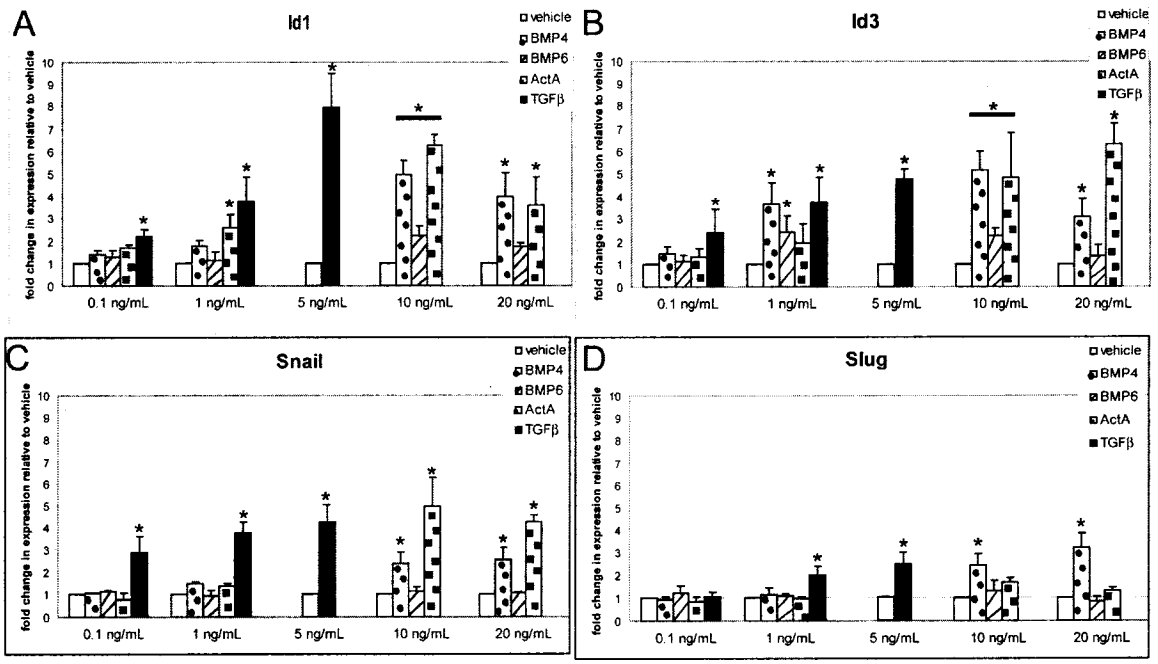


Figure 5-3. TGFβ superfamily ligands differentially modulate Id1, Id3, Snail and Slug mRNA expression. (A-D) Mean mRNA expression data from 3 primary OvCa cell patient samples that were treated with vehicle (0.1% BSA/PBS; clear), BMP4 (circle), BMP6 (hatched), Activin A (squares) from 0.1 ng/mL to 20 ng/mL, or TGFß1 (black) from 0.1 ng/mL to 5 ng/mL. The mRNA of two TGFß signalling target genes, **(A)** Id1 and **(B)** Id3, as well as the transcription factors **(C)** Snail and **(D)** Slug, were assessed in response to 2h of ligand treatment. Data are shown as fold change in expression between ligand-treated relative to vehicle-treated cells. Bars represent standard deviation from three independent experiments conducted in duplicate. $*P < 0.05$.

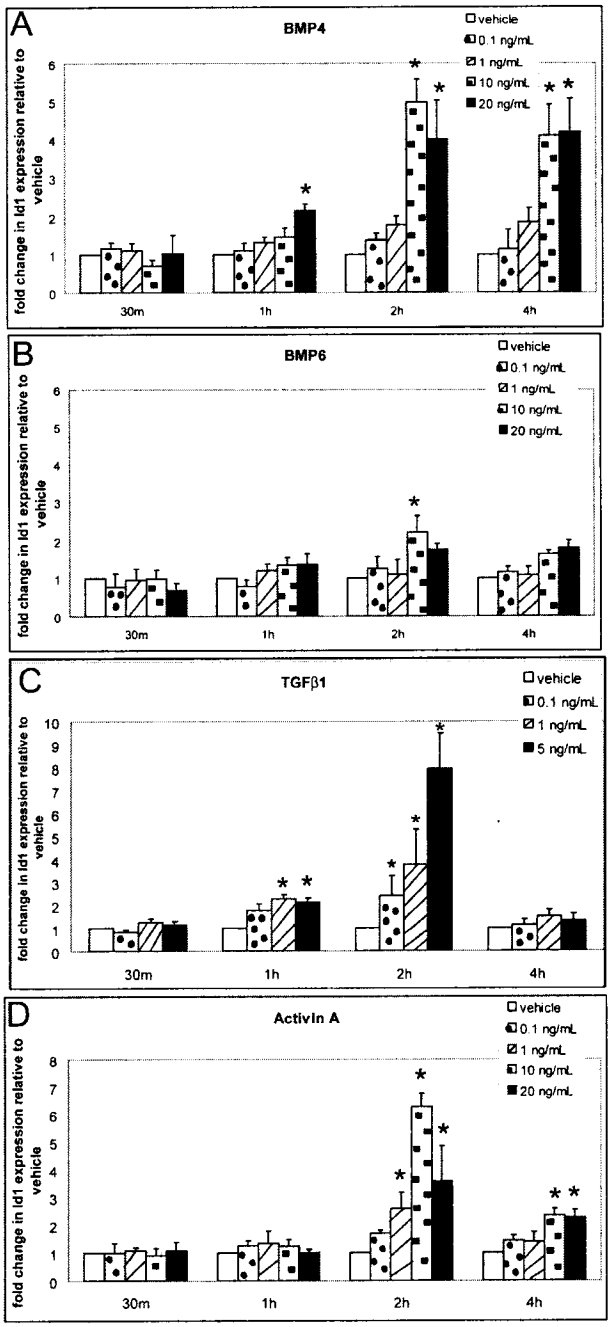


Figure 5-4. TGFβ superfamily ligands modulate Id1 target gene expression in OvCa cells. (A-D) Mean mRNA expression data for Id1 from 3 primary OvCa patient samples that were treated with vehicle (0.1% BSA/PBS; clear) and various doses of **(A)** BMP4 and **(B)** BMP6 (0.1 ng/mL, circle, 1 ng/mL, hatched; 10 ng/mL, squares; and 20 ng/mL, black), **(C)** TGFβ1 (0.1 ng/mL, circle; 1 ng/mL, hatched; and 5 ng/mL, black), and **(D)** Activin A (0.1 ng/mL, circle, 1 ng/mL, hatched; 10 ng/mL, squares; and 20 ng/mL, black) for 30 m, 1, 2 and 4 h treatment periods. Data are shown as fold change in expression between ligand-treated relative to vehicle-treated cells. Bars represent standard deviation from three independent experiments conducted in duplicate. * $P <$ 0.05.

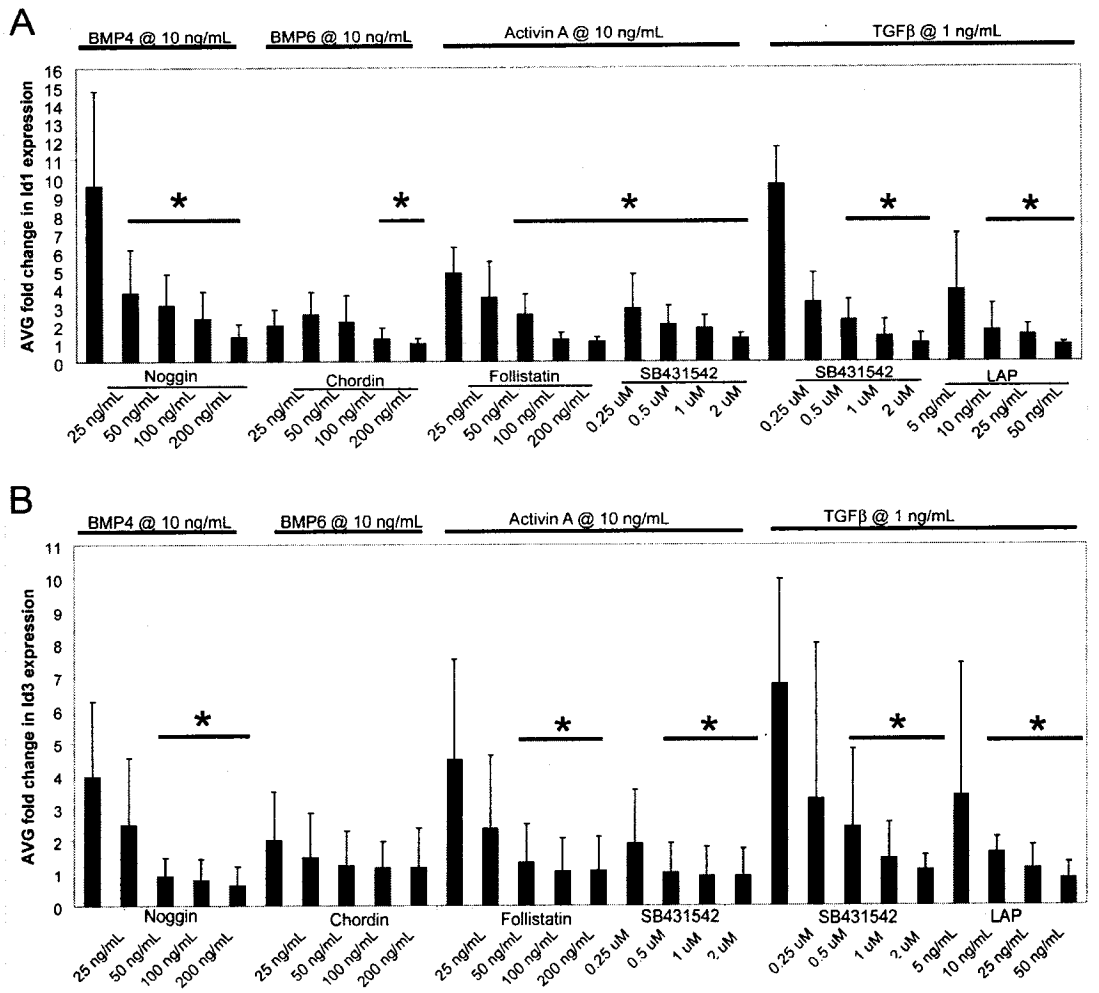


Figure 5-5. TGFβ superfamily inhibitors modulate Id1 and Id3 gene expression in OvCa cells. Mean mRNA expression data from 3 primary OvCa cell patient samples that were treated with vehicle (DMSO), with ligand treatment alone (BMP4, BMP6, Activin A at 10 ng/mL, and TGFß1 at 1 ng/mL) or with ligand plus a range of inhibitor concentrations (BMP4 at 10 ng/mL plus noggin/Fc at 25, 50, 100 and 200 ng/mL; BMP6 at 10 ng/mL plus Chordin at 25, 50, 100 and 200 ng/mL; Activin A at 10 ng/mL plus Follistatin at 25, 50, 100 and 200 ng/mL; Activin A at 10 ng/mL plus SB431542 at 0.25, 0.5, 1 and 2 μM; TGFß1 at 1 ng/mL plus SB431542 at 0.25, 0.5, 1 and 2 μM; or TGFß1 at 1 ng/mL plus LAP at 5, 10, 25 and 50 ng/mL). **(A)** Id1 and **(B)** Id3 were assessed in response to 90 minutes of ligand/inhibitor treatment. Data are shown as fold change in expression between ligand-treated relative to vehicle-treated cells. Bars represent standard deviation from three independent experiments conducted in duplicate. $*P < 0.05$.

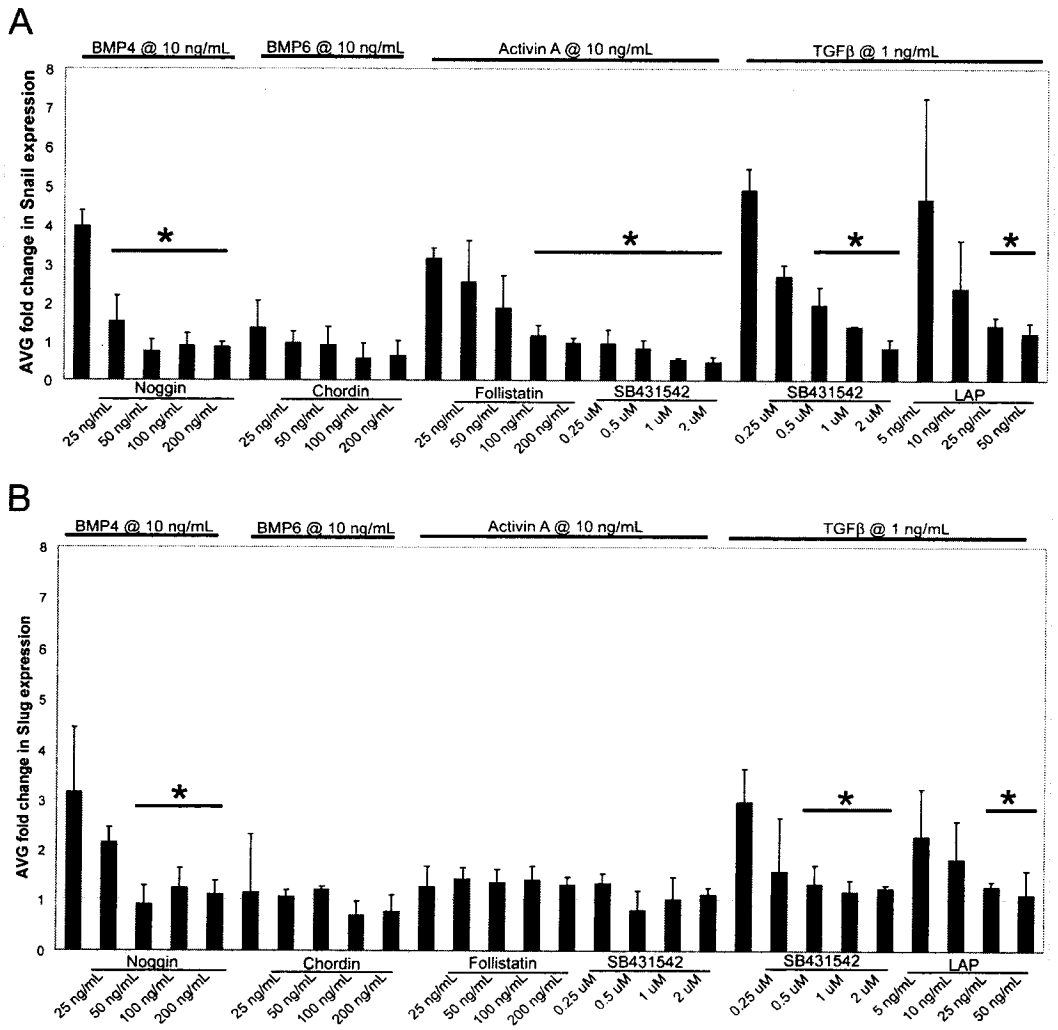
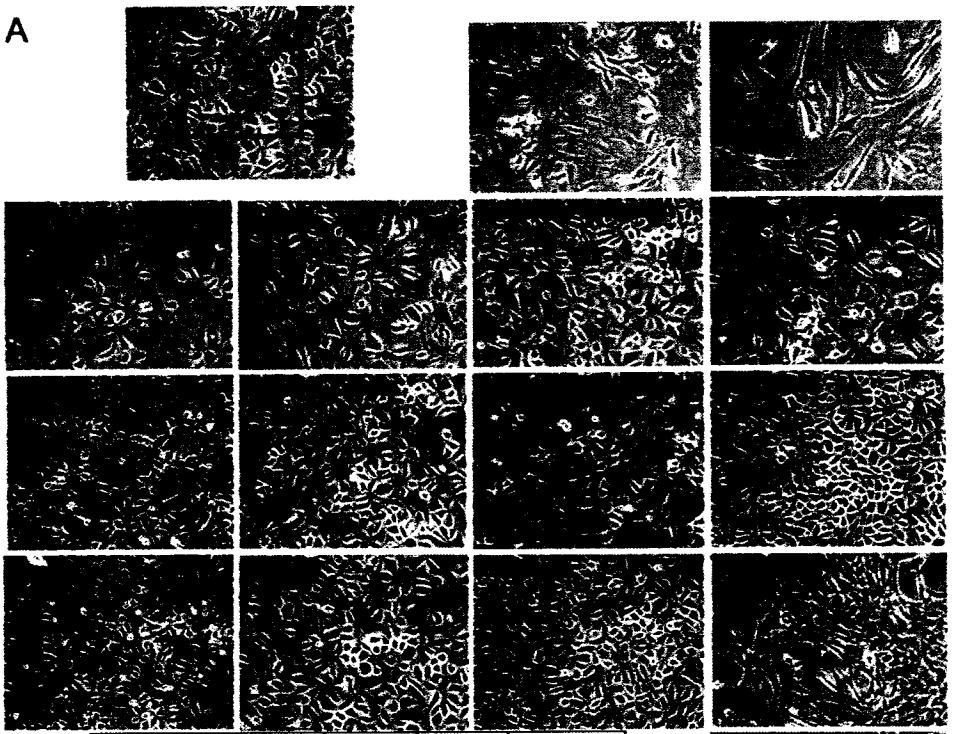


Figure 5-6. TGFβ superfamily inhibitors modulate Snail and Slug gene expression in OvCa cells. Mean mRNA expression data from 3 primary OvCa cell patient samples that were treated with vehicle (DMSO), with ligand treatment alone (BMP4, BMP6, Activin A at 10 ng/mL, and TGFß1 at 1 ng/mL) or with ligand plus a range of inhibitor concentrations (BMP4 at 10 ng/mL plus noggin/Fc at 25, 50, 100 and 200 ng/mL; BMP6 at 10 ng/mL plus Chordin at 25, 50, 100 and 200 ng/mL; Activin A at 10 ng/mL plus Follistatin at 25, 50, 100 and 200 ng/mL; Activin A at 10 ng/mL plus SB431542 at 0.25, 0.5, 1 and 2 μM; TGFß1 at 1 ng/mL plus SB431542 at 0.25, 0.5, 1 and 2 μM; or TGFß1 at 1 ng/mL plus LAP at 5, 10, 25 and 50 ng/mL). **(A)** Snail and **(B)** Slug were assessed in response to 90 minutes of ligand/inhibitor treatment. Data are shown as fold change in expression between ligand-treated relative to vehicle-treated cells. Bars represent standard deviation from three independent experiments conducted in duplicate. $*P < 0.05$.

A



B

Treatment	OvCa # 85	OvCa # 101	OvCa # 111	AVG fold change
	Number of cell bodies			
Vehicle	60	54	45	
BMP4	32	34	28	0.60
BMP4 + Noggin	59	52	44	0.97
Noggin	80	85	61	1.42
BMP6	34	35	31	0.63
BMP6 + Chordin	61	56	42	1.00
Chordin	60	37	44	0.89
Activin A	18	22	18	0.37
Activin A + Follistatin	55	56	47	1.00
Activin A + SB431542	57	54	48	1.01
Follistatin	94	87	81	1.66
TGFB1	11	21	19	0.33
TGFß1 + SB431542	39	49	42	0.83
SB431542	108	101	99	1.96
TGFß1 + LAP	57	38	30	0.77
LAP	87	94	82	1.67

C

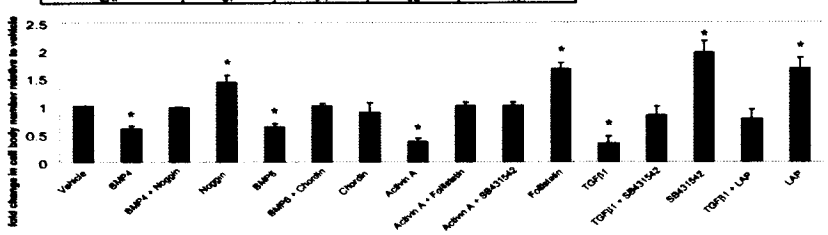


Figure 5-7. TGFß superfamily signalling alters OvCa cellular morphology. **(A)** OvCa cells were treated with various ligands and inhibitors, and phase-contrast images were taken at 6 day post-treatment. Similar results were obtained using primary cells from 3 OvCa samples. Magnification, 100X. **(B)** Cellular density was calculated by counting the number of cell bodies within a defined space (rectangle) for all treatment conditions. 3 OvCa patient samples (OvCa # 85, 101 and 111) were assessed. **(C)** Graph representing the fold changes in cell density calculated by comparing the treatment condition cell density to the vehicle-treated cell density from 3 OvCa patient samples. Bars represent mean data from 3 OvCa patient samples. $*P < 0.05$.

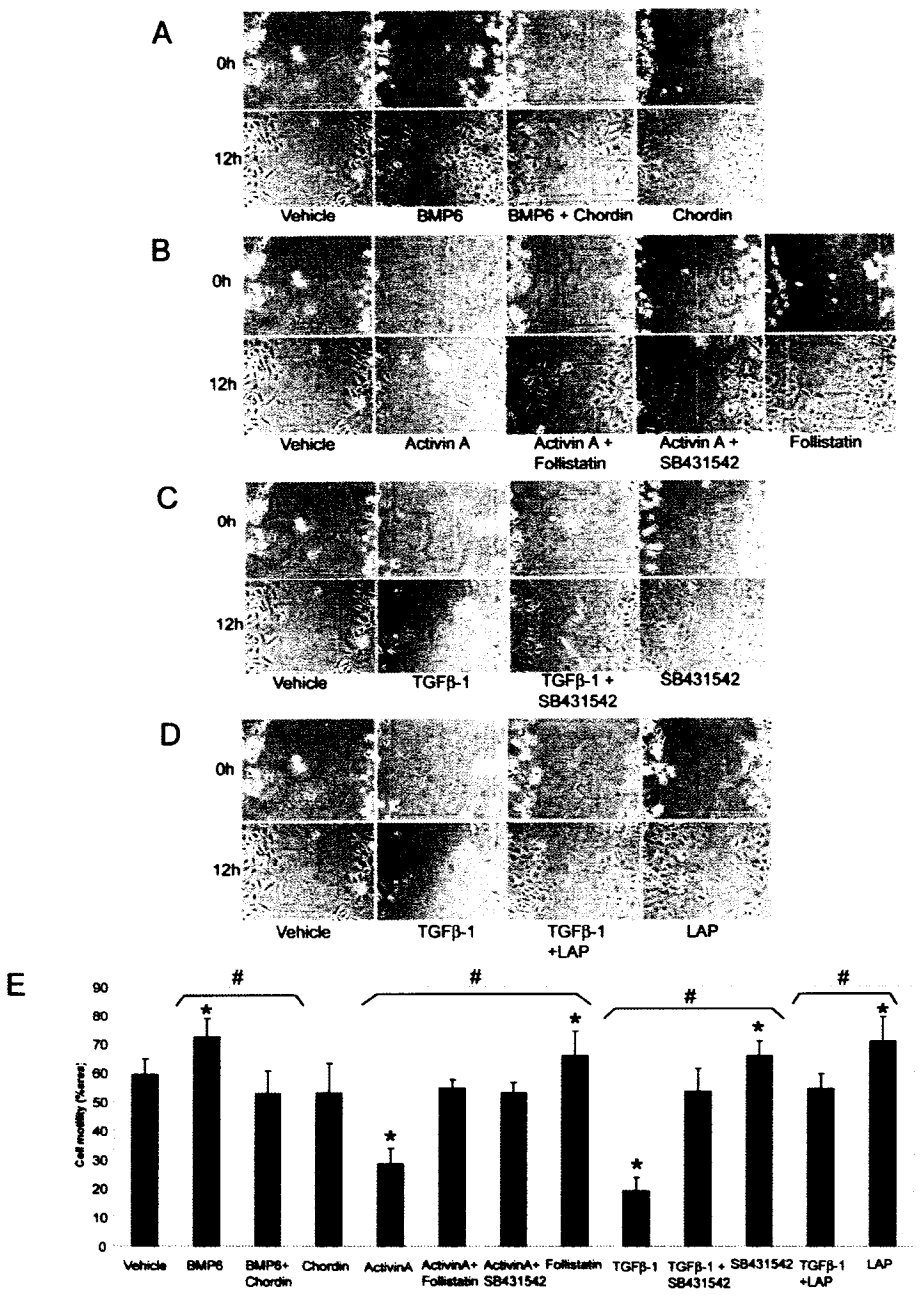


Figure 5-8. Autocrine TGFß superfamily signalling differentially regulates OvCa cellular motility. (A-D) OvCa cells were cultured onto etched grid coverslips, pre-treated with various ligands and inhibitors for 48h, and a wounding assay was performed. The wound was monitored for a period of 12h post-wound. Magnification, 100X. **(E)** The percentage of the total area covered by OvCa cells was assessed using image analysis software (Image J). The graph depicts mean data from one patient sample. Similar results were obtained using primary cells from 3 additional patient samples. Bars represent the standard deviation from three independent experiments conducted in triplicate. $*P < 0.05$, showing a significant difference compared to vehicle-treated cells; # $P < 0.05$, showing a significant difference compared to treated cells within each treatment grouping.

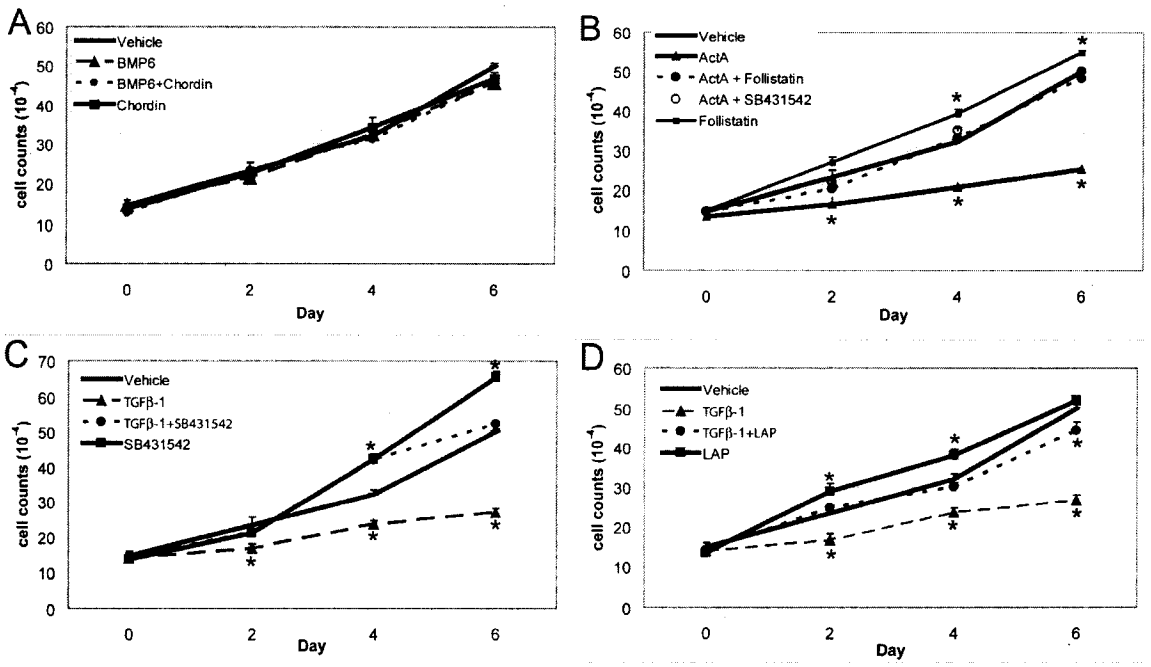


Figure 5-9. Autocrine TGFß superfamily signalling differentially regulates OvCa cellular growth. (A-D) OvCa cells were seeded at low density (2 x 10^4 cells/mL) into 6-well dishes and treated with various ligands and inhibitors for a total period of 6 days, where cell counts were measured every 2 days after treatment. Graphs represent mean data from one representative OvCa patient sample, showing total cell counts at day 0, 2, 4, and 6 post-treatment. Each of the 5 patient samples that were tested were assessed in three independent experiments, each conducted in triplicate and showing similar results. Bars represent standard deviation from three independent experiments conducted in triplicate. $*P < 0.05$.

CHAPTER 6 – DISCUSSION

6.1 GENERAL DISCUSSION

This study initially investigated the impact of BMP4 signalling on primary human OvCa cell biology that subsequently branched into evaluating other members of the TGFß superfamily. The outcome of this research was to reveal the influence of autocrine TGFß superfamily signalling in controlling OvCa cell morphology, adhesion, motility and growth. These results revealed potentially antagonistic cellular effects between BMP4 and TGFß1/Activin A signalling in OvCa cells, where autocrine BMP signalling promotes pro-metastatic behaviours by increasing cellular motility and adhesion, while autocrine TGFß/Activin signalling promotes anti-metastatic behaviours by inhibiting growth and motility. Based on the influence of these signalling pathways on *in vitro* OSE and OvCa cellular behaviours, these antagonistic pathways may exist in a balance that is tightly controlled in normal or pre-neoplastic OSE cells. However, this balance may be altered to favor autocrine BMP signalling influences during OvCa progression, which may promote enhanced OvCa cell adhesion, motility, invasion and metastatic spread.

The study of BMP4 signalling in normal OSE cell behaviour has revealed some interesting insights into its potential role in wound repair following ovulation. Through the induction of an *in vitro* wound which mimics the physical disruption of the OSE monolayer surrounding the ovary following ovulation, cellular motility in terms of wound closure was assessed in response to BMP4 or noggin/Fc treatment. Although the cells were motile and eventually closed the wound, neither BMP4 nor noggin/Fc were able to alter cellular motility, suggesting that BMP4 may not enhance normal OSE motility. However, BMP4 treatment of normal OSE cells does result in similar morphologic changes as with the BMP4-induced cellular remodeling in OvCa cells, suggesting that

BMP4 has the capacity to alter the interactions of OSE cells with their extracellular environment, and thus may still have a role to play in the post-ovulatory repair of the OSE perhaps through the alteration of its adhesive capabilities or through the enhancement of proliferation (Erickson and Shimasaki, 2003). Due to the limited availability of normal OSE samples for analysis, the specific role BMP4 has to play in the post-ovulatory repair of the OSE layer still remains to be determined. My results suggest that a strict regulation of the effects of BMP4 signalling on the OSE exists and that regulation may be lost during OvCa progression, as evidenced by the cellular signalling and behavioural remodeling that occurs in metastatic OvCa cells in response to BMP4. Indeed, our laboratory has shown that in addition to differential motility, primary OvCa cells respond more strongly to BMP4 stimulation by upregulating target genes to a higher degree as compared to normal OSE (Shepherd and Nachtigal, 2003; Theriault et al., 2007), suggesting that metastatic OvCa cells have acquired this enhanced BMP responsiveness. The fact that these BMP4-induced cellular behaviours are seen in a number of OvCa histological subtypes (serous, endometrioid, mucinous; Appendix 1) indicates that this signalling pathway may be a crucial player in the regulation of OvCa progression. TGFß1 and Activin A signalling also cause morphologic changes in OSE cells (data not shown), but induce growth inhibition in both normal OSE and OvCa cells (Auersperg et al., 2001), indicating that the effects of these signalling pathways may remain very similar during the progression from OSE to OvCa, and that other pathways, such as autocrine BMP signalling become dysregulated and may exert a stronger influence on OvCa cells. Therefore one can speculate that a tight balance in the influences of autocrine BMP, TGFß and Activin signalling in the OSE is present and is

responsible for controlling the post-ovulatory repair of the OSE, and that for reasons that are still unknown, the regulation of this signalling balance becomes altered to favor BMP-induced OvCa metastatic cellular behaviours at the expense of TGFß and Activin's signalling effects on motility and growth. A summary of TGFß superfamily ligand effects on normal OSE and OvCa cells is provided in Table 6-1. It is well known that the proper regulation of the OSE's biological functions depends in part on interactions with the underlying stroma through its permeable basement membrane (Auersperg et al., 2001). This interaction is particularly evident during ovulation, where growth factors, hormones and cytokines produced by the stroma will influence the behaviour of OSE cells during and post-ovulation. Thus it is most likely that the stroma may also play a role during OvCa pathogenesis in altering the cell's responses to autocrine and/or paracrine stimulation. Indeed, studies performed in breast, prostate and colon cancers show that factors produced or extracellular interactions provided by the underlying stroma (such as TGFß, EGF, HGF, PDGF as well as ECM and MMP production) affect the proliferative and invasive responses of associated epithelial cells to autocrine and/or paracrine stimulation with the end result of cancer progression (Pardali and Moustakas, 2007).

Other growth factors such as EGF have been shown to counteract the growth-inhibitory effects of TGFß signalling in primary OvCa cells and induce cellular proliferation of TGFß-treated cells (Dunfield and Nachtigal, 2003), suggesting that a number of different growth factor signalling pathways may act in concert to exert tumor-promoting effects and confer resistance to tumor-suppressing effects. In fact, a recent study revealed the synergistic effect of EGF and HGF signalling on increasing cellular

invasion in OvCa cell lines, highlighting the involvement of many different growth factors participating in networks of signalling cross-talk during the promotion of OvCa tumorigenesis (Zhou et al., 2007). These results do not completely discount the influence of TGFß and Activin signalling, as these signalling pathways produce immune-modulating effects in cancer cells that may favor tumor cell escape from immune surveillance (Wong and Leung, 2007). However, this thesis offers evidence that autocrine BMP signalling provides an alternative pathway through which the disruption of normal growth factor signalling processes in the OSE may contribute to the tumorigenic progression of OvCa.

Based on the results obtained from this study, a model of OvCa cell progression can be proposed (Figure 6-1), where during tumorigenesis, OvCa cells become more sensitive to the influence of autocrine BMP signalling as compared to TGFß or Activin signalling, and that this enhanced capability of responding to BMP will induce the OvCa tumor cells to undergo an EMT. The induction of an EMT response through the upregulation of Snail and Slug, and down-regulation of E-cadherin will cause the tumor cells to disaggregate, become more motile through the upregulation of Rho-GTPases and disseminate into the peritoneal cavity. The fact that primary OvCa cells maintain an autocrine BMP signalling loop (Shepherd and Nachtigal, 2003) indicates that the BMP stimulus may be continuously replenished, allowing for the OvCa cells to be influenced by BMP even when exposed to different extracellular environments. The enhanced adhesive properties induced by BMP signalling through increased integrin receptor, ECM and FAP expression will then promote the reattachment of the metastasized OvCa cells to peritoneal surfaces, where other mitogenic growth factors produced by the cancer cells,

present in the ascitic fluid, or produced by the cells at the attachment site such as EGF and HGF, will act in concert with BMP to promote immune evasion, tumor cell attachment and metastatic tumor invasion and growth. Recent reports have highlighted the importance of EMT-MET interconversions in the process of OvCa metastasis, where OvCa cells undergo an EMT to detach from the primary tumor and disseminate throughout the peritoneal cavity, followed by an MET to reattach to extra-ovarian surfaces (Ahmed et al., 2007). Although exogenous BMP4 stimulation induced an irreversible EMT remodeling response in primary OvCa cells (this thesis), there is a possibility that autocrine BMP signalling may not induce such a dramatic response. Indeed, blocking autocrine BMP signalling through treatment of OvCa cells with noggin/Fc further decreases cellular motility, adhesion and induces the cells to acquire a more epithelial-like phenotype, suggesting that OvCa cells remain plastic to the influences that may be present in the extracellular environment, and may undergo a MET. Therefore it is most likely that although autocrine BMP signalling is continuously present in OvCa cells and induces increased motile and adhesive capabilities allowing for OvCa cell seeding onto peritoneal surfaces, some of the BMP-induced signalling responses such as EMT may be altered by other growth factors in the ascites or peritoneal surfaces such as EGF or HGF that could change the behaviour of the OvCa cells to proliferate and invade on extra-ovarian surfaces. TGFß and Activin signalling, although not able to counteract the effects of BMP signalling on OvCa cells, may also confer immune-evasive capabilities through autocrine and/or paracrine mechanisms in order to provide an immunologically privileged environment while in transit through the peritoneal cavity, as well as when adhered to extra-ovarian surfaces. Indeed, our laboratory has determined

that TGFß is present in ascites fluid from OvCa patients at levels well above the threshold of the TGFß receptor complex (2.3 ng/ml; unpublished result). This model thus points to autocrine BMP signalling as a major contributor to OvCa metastasis, however the ultimate impact of this signalling pathway in combination with other known modulators of OvCa cell behaviour remains to be determined.

The mechanisms through which OvCa cells acquire sensitivity to BMP signalling are currently unknown, but may lie within the accumulation of genetic abnormalities that are proposed to occur during post-ovulatory OSE repair and leading to the formation of OvCa. Proteins that regulate signal transduction pathways such as PI3K, as well as growth factor receptor signalling including TßRI, TßRII and Smad 2 have all been shown to be mutated in OvCa (Bilanges and Stokoe, 2007; Levy and Hill, 2006; Samuels and Ericson, 2006; Wang et al., 2000; Zhang et al., 2007). Recently, a genomic analysis of normal and cancerous ovarian tissues revealed that PI3K mutations were commonly present in OvCa tissues as compared to normal OSE cells (Zhang et al., 2007), suggesting that PI3K activity may be enhanced in OvCa. PI3K has been shown to be activated downstream of BMP signalling (Langenfeld et al., 2005), therefore BMP-induced effects may be enhanced in OvCa cells through overactive PI3K expression. TGFß and Activin signalling can also activate PI3K (Dupont et al., 2003; Parsons et al., 2007), but in contrast to BMP receptor signalling, mutations present in TGFß and/or Activin signalling components in cancer cells may decrease PI3K-dependent TGFß and/or Activin signalling processes (Levy and Hill, 2006; Risbridger et al., 2001). Therefore the combination of PI3K mutations increasing PI3K expression, as well as mutations in TGFß and Activin signalling pathways that may decrease their signalling capacity, could

be present in OvCa cells and may account for the enhanced BMP signalling activity seen in OvCa cells. In fact, we have determined that the mRNA for BMPRII and ALK3, and Smad 1 and Smad 5 protein levels remain unchanged between OSE and OvCa cells (BLT, TGS, MWN, unpublished observations); as a result, elevated receptor and/or intracellular Smad expression is unlikely to account for elevated BMP responses in OvCa cells. Further investigation is thus warranted to study the implications of these mutations in TGFß superfamily signalling-induced OvCa cell behaviour.

6.2 SIGNIFICANCE

OvCa is a disease that is to this day undetectable at early stages, partly due to the lack of understanding of how this disease develops. Although a number of promising therapies are being tested to treat recurring disease, the prognosis of OvCa who present with recurrent OvCa remains in general very poor. Since the majority of OvCas arise from the OSE, an understanding of the processes that govern the biology of both of these cells types is fundamental in designing new therapeutic approaches. This study revealed insights into the regulation of normal OSE and OvCa cell biology in response to autocrine BMP, Activin and TGFß signalling.

The culture of primary human normal OSE cells isolated from normal ovarian tissue and OvCa cells isolated from the ascites of patients were used as model systems to study the *in vitro* effect of BMP signalling on their cellular behaviours. The isolation techniques and culture conditions favorably support the growth of these cells. There is however the potential for contamination of these primary cultures by other cell types including fibroblasts, which form the stroma of many organs contained within the

abdominal cavity, and mesothelial cells, which line the peritoneal wall and could be contained within the ascitic fluid. It was therefore very important to confirm the identity and purity of the primary cultures that were being used in this study. Chapter 3 describes an immunocytochemical and QPCR procedure that has been developed to confirm the identity and purity of the OSE and OvCa cells that our laboratory obtains. Through the differential expression of 6 different markers, one can effectively confirm the nature of the cells being cultured, as well as identify any contaminating non-epithelial cell types that could be present within these cultures. It was therefore crucial to identify the nature of these primary cultures in order to draw comparative conclusions about the behaviour of these cells.

This body of work revealed the contributions of autocrine BMP, TGFß and Activin signalling in OvCa cell behaviours. Autocrine BMP signalling has a tumor-promoting effect through the enhancement of cellular adhesion, motility and invasion, while autocrine TGFß and Activin signalling have tumor-suppressing effects through the reduction of cellular motility and growth inhibition. These results suggest a model of OvCa progression where OvCa cells acquire an enhanced sensitivity to the influence of autocrine BMP signalling as compared to TGFß or Activin signalling, and that this enhanced capability of responding to BMP may partly drive OvCa metastasis. Thus BMP signalling may be crucial player in the regulation of OvCa progression. OvCa cells still have the capacity to respond to TGFß and Activin signalling *in vitro* through the induction of growth and motility inhibition, therefore either blocking BMP responsiveness or enhancing TGFß/Activin signalling may prove useful in the attempt to halt or stop OvCa metastatic progression. This thesis has shown that the endogenously-

expressed inhibitor Noggin can block the effects of exogenous and endogenous BMP signalling in OvCa cells and underscores the potential application of this BMP inhibitor as a therapy for the treatment of OvCa. Indeed, Noggin has been proposed as a therapy to block metastatic progression to bone in prostate cancer (Haudenschild et al., 2004; Schwaninger et al., 2007). Furthermore, the fact that Noggin showed no effect on normal OSE cells in terms of cell motility highlights the potential specificity of Noggin for OvCa cells, an attractive characteristic for any cancer treatment. Blocking BMP signalling or enhancing TGFß/Activin responsiveness in OvCa cells could be provided by promoting the expression of either Noggin, TGFß/Activin ligands and/or receptors through the use of gene therapy. OvCa is often confined to the peritoneal cavity even when metastatic spread has occurred, making this type of therapy attractive for local delivery and action. Adenoviral vectors have shown promise in effectively delivering and expressing genetic material using OvCa tumor-specific promoters, with few documented incidences of toxicity in Phase I and II clinical trials (Raki et al., 2006). This thesis lays the groundwork to determine whether targeting the BMP signalling pathway may constitute a viable therapeutic option for women suffering from OvCa.

6.3 FUTURE DIRECTIONS FOR THIS RESEARCH

6.3.1 The Role Of TGFß Superfamily Signalling In Normal OSE

The characterization of the effects of TGFß superfamily signalling on normal OSE cells in culture is crucial in broadening the understanding of how these signalling pathways may be involved in OSE biology and in the neoplastic transformation of the OSE. By comparing the behavioural responses between normal OSE cells and OvCa

cells through the assessment of growth, motility, adhesion and invasion, additional differences may be identified that support the involvement of an altered balance of TGFß superfamily signalling in OvCa progression.

6.3.2 Intracellular Mediators Involved In BMP4-Induced Effects

Although some of the BMP4-induced effects such as morphology and motility can be attributed to Smad-dependent signalling, there is some evidence that Smad-independent early signalling events such as the transient activation of the Rho-GTPases seen in OvCa cells may also contribute to the cellular remodeling that is exhibited by OvCa cells, suggesting that both Smad-dependent and Smad-independent signalling may play a role in BMP4-induced changes in OvCa behaviour. Therefore a dualistic model of intracellular mediator activation could be proposed where the immediate, Smad-independent activation of signalling molecules may be linked to the simultaneous activation of Smad phosphorylation, leading to the subsequent BMP4-induced cellular remodeling and behavioural changes seen in OvCa cells. p38 MAPK, ERK1/2, PI3K, phospholipid balance and Rho-GTPases have all been involved in BMP-induced signalling effects, which can be Smad-dependent or Smad-independent (Chan et al., 2007; Foletta et al., 2003; Hartung et al., 2006; Langenfeld et al., 2005; Theriault et al., 2007); therefore the activation of these individual molecules could be examined following BMP4 treatment of OvCa cells, to reveal the potential involvement of these mediators downstream of BMP receptor activation, but upstream of the changes in cellular behaviour. The use of small molecule inhibitors, shRNA or adenoviral expression systems expressing dominant-negative versions of these molecules could also be used in the presence or absence of BMP4 stimulation to evaluate the contribution of

these mediators in the BMP4-induced cellular behaviours. Furthermore, the dependence upon Smad activity for the activation of these aforementioned molecules can be assessed through shRNA-mediated Smad knockdown. These experiments would highlight the link between Smad-dependent and Smad-independent signalling mechanisms that occurs immediately following BMP4 stimulation, leading to the BMP4-induced cellular remodeling of OvCa cells.

6.3.3 Synergism/Antagonism Of BMP And TGFß/Activin Signalling

This work assessed the individual contributions of BMP, TGFß and Activin signalling in OvCa cell biology. But it is well documented that all of these pathways are intact in normal OSE and OvCa cells (Dunfield et al., 2002a; Shepherd and Nachtigal, 2003; Steller et al., 2005; Theriault et al., 2007; Wong and Leung, 2007), and therefore the combined effect of all of these ligands should be assessed to determine if these pathways are synergistic or antagonistic in remodeling OvCa cell behaviour. Treatment of OvCa cells with different doses and combinations of the ligands and assessing the resulting cellular effects may prove useful in defining whether the balance of TGFß superfamily signalling is altered in OvCa cells, and if this balance contributes to OvCa metastatic progression. Indeed, our laboratory has determined that co-treatment of primary OvCa cells with TGFß1 and EGF results in decreased growth inhibition induced by TGFß1(Dunfield and Nachtigal, 2003). In addition to assessing these signalling influences in a culture dish, it would also be important to assess the influences of TGFß superfamily ligands in a 3-dimensional spheroid culture system to approximate the *in vivo* environment in an *in vitro* setting, and determine whether these ligands produce the same effects in a 3-dimensional culture system as compared to a 2-dimensional culture

system. Preliminary work not included in this thesis indicates that primary OvCa cell spheroids transmit the BMP4 signal and produce changes in cellular adhesion and motility. These results suggest that autocrine BMP signalling is capable of influencing OvCa behaviour in 3-dimensional spheroids that exist extensively in OvCa patients that develop ascites.

6.3.4 The Role Of TGFß Superfamily Signalling In Vivo

The use of primary cell culture models to study the influence of autocrine signalling has proven invaluable in revealing important aspects of *in vitro* normal OSE and OvCa cell biology. However, the cells being studied normally exist within a 3-dimensional environment, through which the growth conditions in a culture dish cannot exactly replicate. Therefore to fully understand the implications of altered BMP, TGFß and Activin signalling in the formation and progression of OvCa, the results obtained from this study need to be applied to an animal model. The use of recently developed rodent ovarian intrabursal injection techniques (Garson et al., 2005) would prove useful to target and deliver viral agents overexpressing components of the BMP, TGFß and/or Activin signalling pathways to the mouse OSE. Indeed, this technique has been used in our laboratory to deliver Ad-ALK3QD to the bursa of female mice and induce OSE-specific overexpression. These studies are currently ongoing and will evaluate whether an activated BMP signalling pathway will induce pre-neoplastic changes to the mouse OSE. These future studies would reveal the potential *in vivo* roles of TGFß superfamily members in the initiation and spread of OvCa from the OSE, as well as provide the tools for the development and testing of novel therapeutic agents to slow or halt the progression of OvCa.

	OSE	OvCa
Morphology		
BMP4	cell spreading	cell spreading
TGFβ	cell spreading	cell spreading
Activin A	cell spreading	cell spreading
Motility		
BMP4	no effect	increase
TGFβ	n/a	decrease
Activin A	n/a	decrease
Invasion		
BMP4	n/a	increase
TGFβ	n/a	n/a
Activin A	n/a	n/a
Proliferation		
BMP4	n/a	no effect
TGFβ	n/a	decrease
Activin A	n/a	decrease

Table 6-1. Summary of cellular effects induced by TGFβ superfamily signalling.
n/a = not assessed.

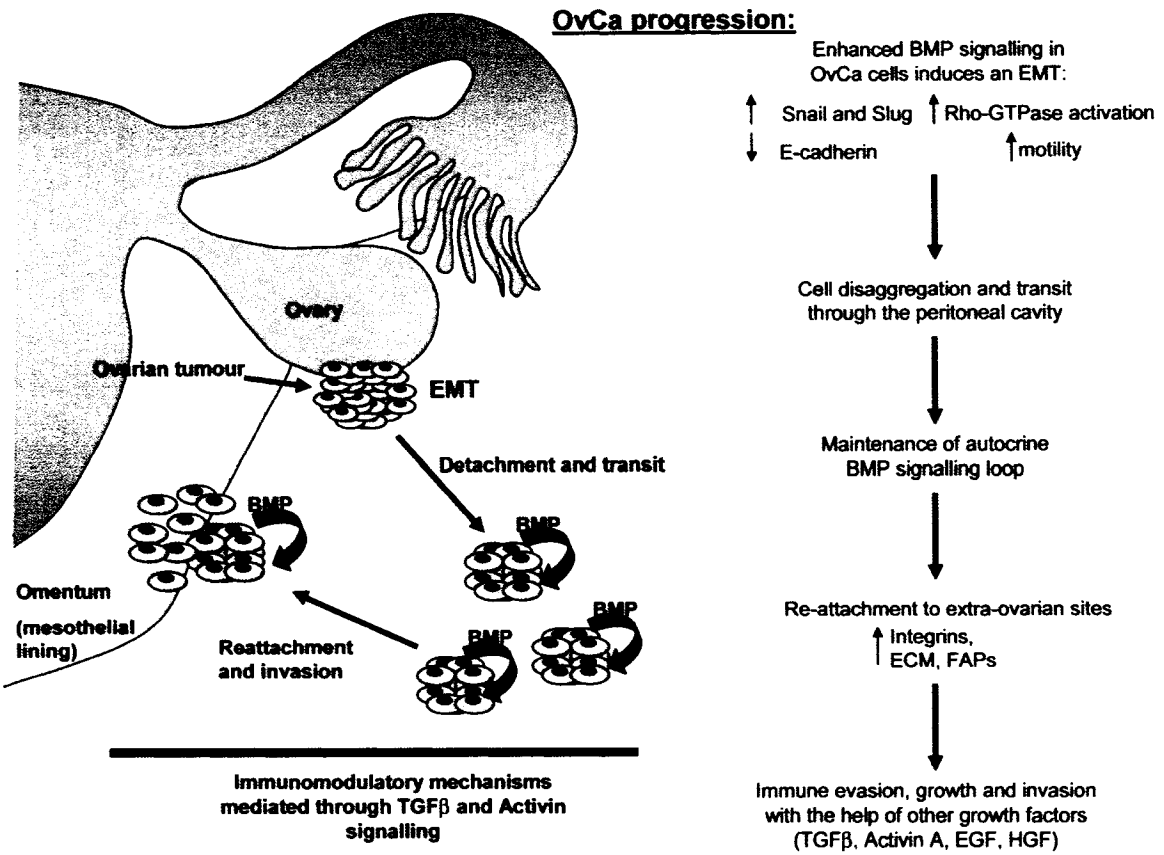


Figure 6-1. Proposed model for OvCa tumor progression and metastasis. During tumorigenesis, OvCa cells become more sensitive to the influence of autocrine BMP signalling, and this will induce the OvCa tumor cells to undergo an EMT. The induction of an EMT response through the upregulation of Snail and Slug, and down-regulation of E-cadherin will cause the tumor cells to disaggregate, become more motile through the upregulation of Rho-GTPases and disseminate into the peritoneal cavity. OvCa cells maintain an autocrine BMP signalling loop, allowing for the OvCa cells to be influenced by BMP even when exposed to different extracellular environments. The enhanced BMP-induced adhesive properties through increased integrin receptor, ECM and FAP expression will promote the reattachment of the metastasized OvCa cells to peritoneal surfaces, where other mitogenic growth factors present in the tumor microenvironment such as EGF and HGF, will act in concert with BMP to promote immune evasion, tumor cell attachment and metastatic tumor invasion and growth. TGFß and Activin A signalling may however confer immune-evasive capabilities through autocrine and/or paracrine mechanisms in order to provide an immunologically privileged environment while in transit through the peritoneal cavity, as well as when adhered to extra-ovarian surfaces.

APPENDIX 1

APPENDIX 1: Tables depicting the types of analyses that were conducted on each OvCa sample, along with some of their diagnoses.

OvCa#	Diagnosis	Stage/Grade	Analyses		
			Cytokeratins	CUTL-1 QPCR	Morphology
28				+	+
30					+
43	serous	n/a			+
44					+
45				+	+
46	serous	n/a		+	+
63					+
64	endometrioid	n/a		+	+
67	uterine papillary serous carcinoma	4B			+
68					+
69				+	+
70				+	+
71	mucinous	n/a		+	+
85	serous	Grade 3/Stage 3C	+		+
87	ovarian adenocarcinoma	n/a		+	+
88	serous	Grade3/Stage 3C			+
89			+		+
90	n/a	Stage 3C			+
91					+
92	serous	Grade 2	+	+	+
101	serous	Grade 2/Stage 1A			+
103	compatible with ovary (previous lymphoma)		+	+	+
111					+

OvCa#	Diagnosis	Stage/Grade	Analyses		
			Cell area/density	Integrin QPCR	Ad-ALK3QD
28					
30				+	
43	serous	n/a		+	+
44					
45					
46	serous	n/a	+	+	
63				+	
64	endometrioid	n/a	+	+	+
67	uterine papillary serous carcinoma	4B		+	
68			+	+	+
69				+	
70				+	
71	mucinous	n/a	+	+	+
85	serous	Grade 3/Stage 3C		+	+
87	ovarian adenocarcinoma	n/a			+
88	serous	Grade3/Stage 3C	+		
89			+		+
90	n/a	Stage 3C			+
91					
92	serous	Grade 2			+
101	serous	Grade 2/Stage 1A	+		+
103	compatible with ovary (previous lymphoma)		+		
111					

OvCa#	Diagnosis	Stage/Grade	Analyses		
			Commitment	SMA	Snail/Slug QPCR
28					
30					
43	serous	n/a			+
44					
45					
46	serous	n/a	+		+
63					
64	endometrioid	n/a	+		+
67	uterine papillary serous carcinoma	4B			
68			+		
69					
70					
71	mucinous	n/a	+		+
85	serous	Grade 3/Stage 3C		+	+
87	ovarian adenocarcinoma	n/a			
88	serous	Grade3/Stage 3C			
89					
90	n/a	Stage 3C			
91					
92	serous	Grade 2			
101	serous	Grade 2/Stage 1A		+	+
103	compatible with ovary (previous lymphoma)				
111					

OvCa#	Diagnosis	Stage/Grade	Analyses		
			Snail/Slug protein	Adhesion	Motility
28					
30					+
43	serous	n/a		+	+
44					
45					+
46	serous	n/a		+	+
63					+
64	endometrioid	n/a		+	+
67	uterine papillary serous carcinoma	4B			
68					+
69				+	+
70					+
71	mucinous	n/a		+	+
85	serous	Grade 3/Stage 3C	+		+
87	ovarian adenocarcinoma	n/a			+
88	serous	Grade3/Stage 3C	+		+
89					+
90	n/a	Stage 3C			+
91			+		+
92	serous	Grade 2			+
101	serous	Grade 2/Stage 1A			+
103	compatible with ovary (previous lymphoma)				+
111					+

OvCa#	Diagnosis	Stage/Grade	Analyses		
			Invasion	Cofilin protein	Rho GTPases
28					
30					
43	serous	n/a	+		
44					
45					
46	serous	n/a			
63					
64	endometrioid	n/a	+	+	+
67	uterine papillary serous carcinoma	4B			
68					
69					
70				+	
71	mucinous	n/a	+	+	
85	serous	Grade 3/Stage 3C	+		+
87	ovarian adenocarcinoma	n/a	+		
88	serous	Grade3/Stage 3C			
89			+		+
90	n/a	Stage 3C	+		
91					
92	serous	Grade 2	+		
101	serous	Grade 2/Stage 1A	+		+
103	compatible with ovary (previous lymphoma)				
111					

OvCa#	Diagnosis	Stage/Grade	Analyses		
			Smad lentivirus	Receptor QPCR	pSmad protein
28					
30					
43	serous	n/a			
44					
45				+	
46	serous	n/a		+	
63				+	
64	endometrioid	n/a		+	
67	uterine papillary serous carcinoma	4B			
68					
69					
70					
71	mucinous	n/a		+	
85	serous	Grade 3/Stage 3C			+
87	ovarian adenocarcinoma	n/a			
88	serous	Grade3/Stage 3C			
89			+		
90	n/a	Stage 3C			
91					+
92	serous	Grade 2			+
101	serous	Grade 2/Stage 1A	+	+	
103	compatible with ovary (previous lymphoma)		+		
111					

OvCa#	Diagnosis	Stage/Grade	Analyses		
			Dose resp ligands QPCR	Dose resp inhibitors QPCR	Growth curves
28					
30					
43	serous	n/a			+
44					+
45					+
46	serous	n/a			
63					
64	endometrioid	n/a			+
67	uterine papillary serous carcinoma	4B			
68					
69					
70					
71	mucinous	n/a			
85	serous	Grade 3/Stage 3C		+	+
87	ovarian adenocarcinoma	n/a			
88	serous	Grade3/Stage 3C	+		
89					
90	n/a	Stage 3C			
91					+
92	serous	Grade 2	+	+	
101	serous	Grade 2/Stage 1A			+
103	compatible with ovary (previous lymphoma)		+	+	
111					+

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