E-CADHERIN REGULATION IN OVARIAN CANCER: MECHANISMS AND POTENTIAL FUNCTIONAL ROLES

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A THESE SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOSPHY

in

The Faculty of Graduate Studies

(Reproductive and Developmental Sciences)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

April 2012

Abstract

E-cadherin is a cell-cell adhesion protein and tumor suppressor that is silenced in many malignancies. However, the role of E-cadherin in ovarian cancer progression is still controversial. In an attempt to define the regulation of E-cadherin in ovarian cancer, we found that local growth factors, FGF2 and IGF1, suppress E-cadherin expression in ovarian cancer cells. To elucidate the role of E-cadherin in ovarian cancer progression, we found that stable knockdown of E-cadherin significantly enhances, whereas overexpression of E-cadherin reduces tumor cell growth and invasion. Loss of E-cadherin results in constitutive activation of phosphoinositide 3-kinase (PI3K)/Akt signaling by inhibition of PTEN transcription through downregulation of Egr1. In addition, immunofluorescence microscopy and TCF promoter/luciferase reporter assays showed that E-cadherin loss was associated with enhanced nuclear β-catenin signaling. Constitutive activation of PI3K/Akt signaling reinforced nuclear β-catenin signaling by inactivating glycogen synthase kinase-3 β indicating cross talk between the PI3K/Akt and β-catenin signaling pathways. Furthermore, we found that E-cadherin negatively regulates tumor cell growth, in part, by positively regulating PTEN expression via β-catenin-mediated Egr1 regulation, thus influencing PI3K/Akt signaling. Finally, the constitutive activation of PI3K/Akt signaling activates its downstream mammalian target of rapamycin (mTOR) signaling pathway. The pharmacological inhibition of PI3K and mTOR suggests that PI3K/Akt/mTOR is required for E-cadherin-depletion-induced tumor cell motility. Moreover, loss of E-cadherin induces tumor cell invasion, in part, by activation of Rho GTPase, Cdc42 and Rac1. In summary, endogenous E-cadherin inhibits PI3K/Akt signaling and Rho GTPase activation. Thus, the loss of E-cadherin itself may contribute to dysregulate PI3K/Akt signaling and Rho GTPase activation to promote tumor proliferation and invasion in human ovarian cancer cells.

Preface

A version of chapter 2 has been submitted. Man-Tat LAU, Peter C.K. LEUNG, Fibroblast growth factor-2 induces E-cadherin down-regulation via PI3K/Akt/mTOR and MAPK/ERK signaling in ovarian cancer cells.

I was responsible for the experimental design and conducted all the experiment in this chapter. I wrote the manuscript which was revised by my supervisors.

A version of chapter 3 has been submitted. Man-Tat LAU, Peter C.K. LEUNG, The PI3K/Akt/mTOR signaling pathway mediates Insulin-like growth factor 1-induced E-cadherin down-regulation and cell proliferation in ovarian cancer cells.

I was responsible for the experimental design and conducted all the experiment in this chapter. I wrote the manuscript which was revised by my supervisors.

A version of chapter 4 has been published. Man-Tat LAU, Christian KLAUSEN and Peter C.K. LEUNG, 2011, E-cadherin inhibits tumor cell growth by suppressing PI3K/Akt signaling via β-catenin-Egr1-mediated PTEN expression. *Oncogene* 30: 2753-66.

I was responsible for the experimental design and conducted all the experiment in this chapter. I wrote the manuscript which was revised by Christian Klausen and my supervisors.

I performed all the experiments in Appendix 1.

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

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ANOVA	Analysis of variance
APC	Adenomatous polyposis coli
CDK	Cyclin-dependent kinase
cDNA	Complementary deoxyribonucleic aicd
DNA	Deoxynucleic acid
dNTP	Deoxynucleoside triphosphate
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGR1	Early growth response 1
EOC	Epithelial ovarian cancer
ERK	Extracellular signal-regulated kinase
ET1	Endothelin 1
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FRS2	Fibroblast growth factor receptor substrate 2
FSH	Follicle stimulating hormone
GAP	GTPase activating protein
GEF	Guanine nucleotide exchange factor
GSK3β	Glycogen synthase kinase 3 beta
HGF	Hepatocyte growth factor
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein

IGFR	Insulin-like growth factor receptor
JNK	Jun N-terminal kinase
kDa	Kilodalton
LEF	Lymphoid enhancer factor
LPA	Lysophosphatidic acid
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloprotease
MDCK	Madin-Darby Canine Kidney Epithelial
MEK	Mitogen-activated protein kinase/ERK kinase
mRNA	Messenger ribonucleic acid
MW	Molecular weight
OSE	Ovarian surface epithelium
PBS	Phosphatase buffered saline
PCR	Polymerase chain reaction
PDK1	Phosphoinositol-dependent protein kinase 1
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol 4, 5 biphosphate
PIP3	Phosphatidylinositol 3,4,5 phosphate
PTEN	Phosphatase and tensin homolog
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcription polymerase chain reaction
STAT	Signal transducer and activator of transcription
Taq	*Thermus acuaticus*
TCF	T-cell factor
TGF β	Transforming growth factor β
TNFα	Tumor necrosis factor α

uPA	Urokinase-type plasminogen activator
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
Wnt	Wingless-type MMTV integration site family
WT1	Wilms tumor 1

Acknowledgements

First of all, I would like to take this opportunity to express my heartfelt thanks to Dr. Peter CK Leung.

Moreover, I would like to thank Dr. Angela Devlin, Dr. Anthony Cheung, Dr. David Huntsman and Dr. Christian Klausen, who are my thesis committee members

Also, I would like to give my special thanks to Dr. Christian Klauseen for his guidance and advice. I am also grateful to my friends and labmates in Dr. Leung's laboratory. And many thanks to Mrs. Roshni Nair for her help in our department.

Finally, I need to give my special thanks to my parents and my wife, Connie, for their support and care.

Dedication

To my parents

1. Introduction

1.1 Ovarian cancer

Ovarian cancer is the leading cause of gynecological cancer-related death among women in developed countries (Cannistra, 2004). Survival rate is close to 90% among ovarian cancer patients diagnosed at an early stage. Nevertheless, due to non-specific symptoms and the lack of efficient screening for the early detection of ovarian malignancies, the majority of ovarian cancer patients (60-65%), which are diagnosed at a late stage with the cancer, have metastasized to the omentum and surrounding abdominal organ surface (Hudson et al., 2008). The survival rate is only 5-10 % among patients diagnosed at late stage, making this the most lethal gynecological cancer (Christian and Thomas, 2001;Hoskins, 1995;Nguyen et al., 1993).

Epidemiological studies have linked numerous factors to an increased risk of ovarian cancer. For example, female infertility is a risk factor for ovarian cancer (Ness et al., 2002). Moreover, it has been suggested that the use of ovulation induction agents (clomiphene or gonadotropins) in the treatment of infertility may also be a risk factor for ovarian cancer (Anderson and Dimitrievich, 1996;Lacey, Jr. et al., 2002;Riman et al., 2002;Rossing et al., 1994;Whittemore et al., 1992) but this remains controversial. Interestingly, pregnancy, oral contraceptive use, hysterectomy, and tubal ligation have protective effects to reduce the risk of ovarian cancer (Ness et al., 2002). This is presumably due to decreased exposure of the ovary to potential carcinogen factors and/or inflammation during ovulation (Kurian et al., 2005;Ness et al., 2001;Parazzini et al., 1993).

Approximately 90% of ovarian cancers are tumors of epithelial origin (carcinomas) while other types can be derived from the germ cells or the hormone-producing stromal cells (Auersperg et al., 2001). Ovarian cancer is often considered clinically as one disease. However,

there is an increasing realization that ovarian carcinoma is a heterogeneous disease. The major morphological subtypes of ovarian carcinoma are serous (70%), clear cell (10%), endometrioid (10%), mucinous (3%). Other subtypes include transitional (1%), undifferentiated and mixed (6%) (McCluggage, 2011). Different morphological subtypes have a different pathogenesis, which associated with particular underlying molecular changes, and have different natural history and prognosis (Gilks, 2004;Shih and Kurman, 2004;Soslow, 2008).

1.1.1Common subtypes of ovarian cancers

1.1.1.1 Serous carcinoma

Ovarian serous carcinoma can be further classified in to two distinct tumor types, low grade and high grade, which have a different behavior, molecular alterations, pathogenesis and prognosis (Gilks, 2004;Ho et al., 2004;Russell and McCluggage, 2004;Shih and Kurman, 2004;Sieben et al., 2004;Singer et al., 2002;Singer et al., 2003;Singer et al., 2005;Soslow, 2008;Vang et al., 2009). When compared to high grade ovarian serous carcinoma, low grade ovarian serous carcinoma is rare and has been proposed to develop slowly from a benign serous cystadenoma via a serous borderline tumor to an invasive low grade serous carcinoma. (McCluggage, 2011). The precursor lesion for high grade ovarian serous carcinoma remains undefined. Initially, ovarian surface epithelium (OSE) or the epithelium of cortical inclusion cysts, which form when OSE cells are embedded in the ovarian stroma, are thought to be an origin site for high grade ovarian serous carcinoma. However, several recent studies have noted that high grade ovarian serous carcinoma likely arises from the distal fimbrial portion of fallopian tube epithelium (Herrington and McCluggage, 2010;Kindelberger et al., 2007;Lee et al., 2006;Lee et al., 2007;Przybycin et al., 2010). Approximately two-thirds of low grade ovarian

serous carcinoma is associated with *KRAS* or *BRAF* mutations (Ho et al., 2004;Russell and McCluggage, 2004;Sieben et al., 2004;Singer et al., 2002;Singer et al., 2003;Singer et al., 2005;Vang et al., 2009). These mutations have also been found in benign and borderline regions within the same neoplasm, indicating these mutations are early event in tumorigenesis. The *ERBB2* gene is also frequently found to be mutated in low grade ovarian serous carcinoma (Vang et al., 2009). In contrast, high grade ovarian serous carcinoma is associated with p53 dysfunction or *TP53* mutation and this occurs early in tumor development (Ho et al., 2004;Russell and McCluggage, 2004;Sieben et al., 2004;Singer et al., 2002;Singer et al., 2003;Singer et al., 2005). High grade ovarian serous carcinoma is also often found with *BRCA1* and *BRCA2* mutation (McCluggage, 2011).

Recently, a new theory has been proposed to explain the development of low- and high-grade serous carcinomas from fallopian tube epithelium (Kurman and Shih, 2010). During ovulation, the fallopian tube fimbria contact the ovarian surface where the OSE is disrupted. It is possible that the fallopian tube epithelium are dislodged and implanted into the ovary develop into an inclusion cyst, which in turn becomes the origin site of ovarian serous carcinoma. In the case of low grade serous carcinoma, *KRAS* or *BRAF* mutations in the cyst lead to tumor transformation from a serous cystadenoma and to become the serous borderline tumor. In contrast, the high grade serous carcinoma derived from the cyst with the *TP53* mutation or p53 dysfunction, which involves a rapid formation of an intraepithelial carcinoma as an intermediate step. Taken together, both low and high grade serous carcinomas develop presumably from fallopian tubal epithelium that are trapped in the ovary and therefore the ovary is involved secondarily (Kurman and Shih, 2010).

1.1.1.2 Clear cell carcinoma

Clear cell carcinomas are typically cystic neoplasms with polypoid masses that protrude into the cyst, often with prominent cell membranes. Most clear cell carcinomas do not express the estrogen receptor (ER), Wilms tumor 1 (WT1) and p53 (McCluggage, 2011). They usually arise from endometriosis and are diagnosed at early stage (stage I or II). However, the details underlying pathogenesis of the clear cell carcinomas remains largely unknown.

1.1.1.3 Endometrioid adenocarcinoma

The majority of ovarian endometrioid adenocarcinomas is diagnosed as low grade carcinoma and usually confined to the ovary, stage I. Approximately 90% of ovarian endometrioid adenocarcinomas are unilateral. Endometrioid adenocarcinomas often developed from endometriosis or a pre-existing borderline adenofibroma (Bell and Kurman, 2000;Stern et al., 2001). *PTEN*, β-catenin, *KRAS* and *PIK3CA* mutations are usually found in ovarian endometrioid adenocarcinomas (McCluggage, 2011). It has been proposed that the origin of endometriosis develops from retrograde menstrual flow. According to this view, both clear cell carcinoma and endometrioid originally develop from endometrial tissue which implanted in the ovary where they undergo the neoplastic process secondarily (Kurman and Shih, 2010).

1.1.1.4 Mucinous

Similar to low grade ovarian serous carcinomas, ovarian mucinous tumors commonly exhibit *KRAS* mutations which appear in the early stage of the development of this carcinoma.

Again, these mutations are also found in benign, borderline, and malignant sites within the same neoplasm (Cuatrecasas et al., 1997;Gemignani et al., 2003;Scott and McCluggage, 2006). Recent studies suggest that mucinous tumors may develop from transitional-type epithelium located in paraovarian region (Kurman and Shih, 2010).

1.1.2 Growth factor regulation of ovarian cancer progression (FGF, IGF)

Accumulating evidence suggests that hormones, growth factors, and cytokines interact to coordinate normal ovarian physiological functions (Wong and Leung, 2007). Given that these factors play key roles in broad cellular events such as proliferation, motility, and differentiation, any dysregulation of the transduction pathways of these factors may contribute to tumor transformation and progression in ovarian cancers.

1.1.2.1 Fibroblast growth factor 2 regulation of ovarian cancer progression

Fibroblast growth factor 2 (FGF2), which is to a member of the FGF family, plays a fundamental role in various biological activities, including cell proliferation, migration, and differentiation (Bottcher and Niehrs, 2005;Chalkiadaki et al., 2009). FGF2 has also been described as an angiogenic factor (Werner and Grose, 2003) and more recently has been shown to contribute to the development of peritioneal metastasis (Sako et al., 2003). Dysregulated FGF signaling is common in many cancers including ovarian cancer (Barton et al., 1997;Davidson et al., 2002;Fujimoto et al., 1997;Turner and Grose, 2010), suggesting that this signaling can promote tumor development and progression.

FGF2 functions in a classic autocrine or paracrine manner (Turner and Grose, 2010).

FGF2 exerts its signaling through four transmembrane tyrosine kinase receptors (FGFR1, FGFR2, FGFR3, and FGFR4). There is also a fifth related receptor (FGFR5 or FGFRL1) that has no intracellular tyrosine kinase domain, and might negatively modulate the signaling (Wiedemann and Trueb, 2000). Following ligand binding, ligand-dependent receptor dimerization results in recruitment and phosphorylation of adaptor protein, FGFR substrate 2 (FRS2), which leads to the activation of various downstream signaling pathways such as the mitogen-activated protein kinase (MAPK)/extracellular signal-related kinase (ERK), and phosphatidylinositol 3-kinase (PI3K)/Akt transduction pathways (Altomare and Testa, 2005;Eswarakumar et al., 2005). FGFRs can also activate several other signaling pathways such as the p38 MAPK and Jun N-terminal kinase (JNK) pathways, signal transducer and activator of transcription (STAT) signaling in a cell type-specific manner (Hart et al., 2000). These context-dependent signaling pathways are likely due to the cell type-specific expression of various receptors, adaptor protein, signaling molecules and transcription factors (Dailey et al., 2005).

In ovarian cancer, the role of FGF2 in tumor development and progression still remains largely unknown. A high levels of FGF2 and its mRNA have been reported in advanced primary ovarian cancers when compared to normal ovaries (Fujimoto et al., 1997). Also, several studies have shown increased plasma levels of FGF2 in ovarian cancer patients (Barton et al., 1997;Dirix et al., 1997;Salgado et al., 2004). However, the role of FGF2 in ovarian cancer progression is still controversial. High levels of cytoplasmic FGF2 in ovarian tumors are associated with reduced tumor aggressiveness and increased survival rates when compared with patients with low levels of FGF2 (Obermair et al., 1998;Secord et al., 2007). By contrast, several *in vitro* studies and the gene expression profiling studies in advanced ovarian cancer reveal that FGF2 functions as an autocrine growth factor for ovarian cancer cell proliferation (Crickard et al.,

1994;De Cecco et al., 2004;Di Blasio et al., 1993), and invasion (Li and Jiang, 2010). Moreover, FGF2 regulates expression of various genes implicated in angiogenesis or metastasis (Billottet et al., 2004;Giavazzi et al., 2003;Strutz et al., 2002;Wu et al., 2008b), suggesting the FGF2 signaling may be a potential therapeutic target.

1.1.2.2 Insulin-like growth factor 1 regulation of ovarian cancer progression

Insulin-like growth factors (IGFs) are a group of systemic and local growth factors with critical roles in regulating cellular functions such as cell growth, proliferation, survival, and metabolism (Yu and Rohan, 2000). The IGF signaling system consists of of two ligands (IGF1 and IGF2), two transmembrane receptors (IGF1R and IGF2R), six IGF binding proteins (IGFBP-1 to IGFBP-6) that control the ligand availability to the receptors, and a group of IGFBP proteases that cleave IGFBP and modulate the action of IGFs (Adhami et al., 2006). Ligand binding of IGF1 and IGF2 to a receptor tyrosine kinase (RTK) IGF1R triggers the activation of the IGF downstream signaling pathways such as the MAPK/ERK, and PI3K/Akt (Adhami et al., 2006;Pollak et al., 2004). On the other hand, IGF2R is a transmembrane gylcoprotein that lacks an intracellular tyrosine kinase domain. Binding of IGF2 to the IGF2R results in internalization of receptor-ligand complexes and subsequent clearance by the lysosome. Thus, IGF2R functions to clear the extracellular IGF2 and to attenuate signaling (Ghosh et al., 2003).

The IGF axis has been implicated in human cancer development and progression. (Pollak et al., 2004). Emerging evidence shows that elevated plasma levels of IGF1 is associated with a higher risk for various cancers including breast, prostate, and lung (Belfiore and Frasca, 2008;Slomiany et al., 2007;Weiss et al., 2007). In addition, it was demonstrated that metastasis of different cancer cells was reduced by inhibition of IGF1R, suggesting the important role of

IGF signaling in cancer progression (Sachdev, 2008;Sachdev and Yee, 2007). Nevertheless, the role of IGF in ovarian cancer is still poorly understood.

It has been shown that all necessary components, including IGF1, IGFBPs, and IGF1R, for an IGF1-mediated autocrine action are expressed in ovarian cancer cells (Beck et al., 1994;Ouban et al., 2003;Weigang et al., 1994;Yee et al., 1989), suggesting an involvement of the IGF axis in ovarian cancer development. Also, IGF1 levels were higher in fluid from invasive malignant cysts compared to cyst fluid in benign tumors (Karasik et al., 1994). Additionally, the high levels of IGF1 and IGF2 in ovarian cancer are associated with an increased risk of disease, disease progression, and poor prognosis (Brokaw et al., 2007;Lu et al., 2006;Lukanova et al., 2002;Sayer et al., 2005;Spentzos et al., 2007). There is accumulating evidence using *in vitro* models that shows the role of IGF in cellular proliferation (Conover et al., 1998;Kalli et al., 2002), invasion (Kalli and Conover, 2003;Wilson et al., 1999), and angiogenesis (Cao et al., 2007). Therefore, these data suggest that IGF system might be involved in ovarian cancer development and progression.

1.1.3 Ras/Raf/MEK/ERK pathway

The Ras/Raf/MEK/ERK pathway plays an important role in diverse cellular events that can control cell cycle progression (Blalock et al., 1999;Malumbres et al., 2000), apoptosis (Zha et al., 1996), and differentiation. Activation of this pathway can be induced by various cytokines, growth factors and hormones. Following binding of ligands to their specific receptors, formation of the Shc/Grb2/SOS coupling complex occurs. Upon stimulation by this complex, the inactive GDP-bound Ras undergoes a conformational change and is converted into an active GTP-bound form. The activated Ras further recruits and activates the protein kinase activity of Raf which in

turn activates mitogen-activated protein kinase/ERK kinase (MEK). Extracellular-signal-regulated kinases 1/2 (ERK1/2) are well-known downstream mediators of Ras/Raf/MEK signaling, and are activated via phosphorylation mediated by MEK. ERKs can be directly or indirectly phosphorylated and activated many transcription factors to regulate their target genes involved in various cellular functions (McCubrey et al., 2007).

Dysregulation of the Ras/Raf/MEK/ERK pathway has been associated with human cancer progression. Mutations at *BRAF* and *KRAS*, which result in constitutive activation of this signaling pathway, have been frequently detected in many cancers including ovarian cancers (Garnett and Marais, 2004;Vang et al., 2009). About 30% of human cancers have constitutively active *RAS* mutations and amplification of *RAS* proto-oncogenes (McCubrey et al., 2007) whereas *BRAF* is mutated in about 7% of all cancers (Garnett and Marais, 2004). However, recent studies reported that *BRAF* is often mutated in certain types of cancer such as colorectal cancer (5-22%), melanoma (27-70%), papillary thyroid cancer (36-53%), and ovarian cancer (30%). (Davies et al., 2002;Fransen et al., 2004;Garnett and Marais, 2004;Libra et al., 2006;McCubrey et al., 2007).

1.1.4 PI3K/Akt pathway

The PI3K/Akt signaling pathway is activated by diverse stimuli including hormones, growth factors, and extracellular matrix components. Upon activation of the receptor by a specific ligand, a tyrosine phosphorylated receptor recruits the p85 regulatory subunit and the catalytic subunit of PI3K to form a complex. Instead, after ligand binding to the cytokine receptor, Shc binds to the activated receptor to form a complex with Grb-2 and Sos, and subsequently activates the downstream mediator, Ras which, in turns, activates the p110 subunit

of PI3K. Activated PI3K switches phosphatidylinositol 4, 5 biphosphate (PIP2) to phosphatidylinositol 3,4,5 phosphate (PIP3) to activate phosphoinositol-dependent protein kinase 1 (PDK1) and then to activate Akt to mediate the various biological effects such as metabolism, apoptosis, and proliferation. On the other hand, the phosphatase and tensin homolog (PTEN) functions as a tumor suppressor by dephosphorylating PIP3 to attenuate PI3K/Akt signaling (Nicholson and Anderson, 2002).

Dysregulation of PI3K/Akt signaling is common in different human cancers. Constitutive activation of this signaling can arise due to mutations in components of the signaling pathway, or amplification of *AKT* genes. Three isoforms of Akt have been identified: Akt1, 2 and 3. Recent studies suggest that this signaling pathway is vital in cell proliferation and cell survival and thereby promoting cancer progression. The *PTEN* gene and *PIK3CA* gene (that encodes p110a subunit of PI3K) are frequently mutated in many human cancers, including ovarian cancer (Engelman et al., 2006;McCluggage, 2011). Amplification of Akt2 has also been found in ovarian cancer (Bellacosa et al., 1995;Cheng et al., 1992), indicating that Akt amplification at least partly contributes to the tumorigenic effect of aberrant PI3K signaling.

1.2 E-Cadherin

E-cadherin belongs to classical cadherin, which is a calcium-dependent cell-cell adhesion transmembrane glycoprotein. The extracellular regions of E-cadherin are responsible for adhesive recognition through homophilic protein-protein interactions with the ectodomains of other cadherins at the surface of the adjacent cells (Leckband and Prakasam, 2006). The cytoplasmic domain of E-cadherin interacts with various molecules that connect the cadherin to basic intracellular events such as actin cytoskeleton, cell signaling, and trafficking (Bryant and

Stow, 2004;Mege et al., 2006;Yap and Kovacs, 2003). β-catenin binds directly to the carboxyl termini of E-cadherin, whereas p120-catenin interacts with the membrane-proximal region of the cytoplasmic tail of E-cadherin; α-catenin links the cadherin complex by association with β-catenin to the actin cytoskeleton (Geiger et al., 1995;Kemler, 1993). In addition, there is emerging evidence that a large number of cytoplasmic effectors can interact, either directly or indirectly, to the cytoplasmic tail of cadherins so as to link the cadherin complex to the microtubule network and to several signaling molecules (Perez-Moreno et al., 2003;Yagi and Takeichi, 2000). Thus, E-cadherin has a crucial role in physiological tissue organization.

Several lines of evidence demonstrate that E-cadherin functions as a tumor suppressor. First, E-cadherin dysfunction is a common event in tumor progression (Nollet et al., 1999;Yap, 1998). The loss of E-cadherin function is associated with metastasis, peritoneal dissemination, and poor prognosis in ovarian cancer (Daraï et al., 1997;Hudson et al., 2008;Sawada et al., 2008;Veatch et al., 1994;Yuecheng et al., 2006). Second, both *in vitro* and *in vivo* studies demonstrated that E-cadherin dysfunction promotes tumor invasion and metastasis (Vleminckx et al., 1991;Yuecheng et al., 2006). Given that E-cadherin is known to suppress tumor cell invasion, re-expression of E-cadherin in E-cadherin-deficient carcinomas results in suppression of invasive and aggressive behavior (Gottardi et al., 2001;St Croix et al., 1998;Yanagisawa and Anastasiadis, 2006). Furthermore, numerous cancers are associated with both germline and somatic *CDH1* mutations. The germline E-cadherin mutations in one allele and subsequently somatic loss of heterozygosity of the remaining allele have been found to correlate with tumor invasive and metastatic characteristics (Berx et al., 1995;Berx et al., 1996). The germline mutations have also been found in many families with diffuse gastric carcinoma (Guilford et al., 1998), suggesting these mutations are early events in tumorigenesis.

1.2.1 Regulation of E-cadherin in human

In addition to mutation in the E-cadherin gene (*CDH1*), the loss of E-cadherin expression can also be achieved by the epigenetic silencing of the *CDH1* gene during tumor progression (Graff et al., 1995;Hennig et al., 1995). There is compelling evidence that methylation silencing of gene expression is commonly associated with tumor progression and metastasis (Costello and Plass, 2001;Nass et al., 2000;Tamura et al., 2000). The hypermethylation of the E-cadherin promoter has been reported in many human tumors including bladder (Bornman et al., 2001), breast (Toyooka et al., 2002), cervical (Chen et al., 2003), colorectal (Garinis et al., 2002), gastric (Waki et al., 2002), prostatic (Woodson et al., 2003), as well as oral squamous cell carcinoma (Yeh et al., 2002). Our recent study also demonstrated that inhibition of the p53 tumor suppressor gene induces E-cadherin down-regulation via the E-cadherin promoter methylation in serous borderline ovarian tumors (Cheng et al., 2011).

In human cancer cells, E-cadherin expression is usually suppressed at the transcriptional level (Batlle et al., 2000;Cano et al., 2000;Comijn et al., 2001;Hajra et al., 2002). Several transcription repressors have been identified to target the E-cadherin gene promoter including the zinc-finger-containing proteins (Snail, Slug, ZEB1, and ZEB2), and the helix-loop-helix transcription factor (E12/E47, and Twist) via their interaction with the E-box binding site in the promoter (Batlle et al., 2000;Bolos et al., 2003;Cano et al., 2000;Grooteclaes and Frisch, 2000;Peinado et al., 2007). Other mechanisms have been demonstrated to reduce E-cadherin protein level via proteolytic degradation by matrix metalloproteases (MMPs) (Nawrocki-Raby et al., 2003).

1.2.2 The role of E-cadherin in tumorigenesis

Although E-cadherin serves as a tumor suppressor protein through inhibition of invasion and metastasis in many cancers, the underlying mechanisms by which loss of E-cadherin function promotes tumor progression is still largely unknown. Previous studies demonstrated that E-cadherin can modulate various intracellular signaling pathways to suppress tumor progression. Several potential mechanisms have been proposed to account for the tumor-suppressive function of E-cadherin: 1) activation of the Wnt/β-catenin signaling pathway; 2) modulation of growth factor signaling; and 3) signaling via Rho GTPases.

1.2.2.1 Activation of the Wnt/β-catenin signaling pathway

Apart from its vital role in formation of the E-cadherin complex, β-catenin also acts independently of cadherin in the canonical Wnt signaling pathway (Bienz and Clevers, 2000;Polakis, 2000). In the absence of Wnt signaling, non-sequestered cytosolic β-catenin is tightly controlled by adenomatous polyposis coli (APC)-axin-Glycogen synthase kinase 3β (GSK3β) complex. Axin binds directly to β-catenin, which is rapidly phosphorylated by GSK3β and subsequently undergoes ubiquitin-proteasomal degradation (Luo and Lin, 2004). Activation of Wnt signaling, upon ligand binding, phosphorylates and inhibits GSK3β activity, thereby accumulating β-catenin in the cytoplasm. Subsequently, it translocates to the nucleus, where it cooperates with transcription factors of the T-cell factor (TCF) family to regulate their target genes, including *CCND1*, *FN1*, *MMP7*, *CD44*, *TCF1* and others, which are mostly involved in cell proliferation as well as tumor progression (Cavallaro and Christofori, 2004).

In the Wnt signaling pathway, β-catenin signaling is a fundamental mediator in post-embryonic development and physiological tissue turnover (Cadigan and Nusse, 1997). Anomalous Wnt pathway has been found to contribute to tumor progression. Indeed, in many sporadic and familial colorectal cancers, either mutations in β-catenin or inactivating mutations in APC, which becomes resistant to phosphorylation by GSK3β, lead to hyperactivation of Wnt signaling. The aberrant β-catenin pathway activation is also commonly found in breast, skin and heopoietic tumors (Fodde and Brabletz, 2007).

Since β-catenin is a common component for cadherin complex and for the induction of TCF-dependent target genes, many studies have focused on the relationship between cadherins and Wnt/β-catenin signaling. It has been shown that sequestration of β-catenin, by E-cadherin at the membrane, antagonizes Wnt signaling by competing with the β-catenin/TCF-mediated transcriptional regulation (Jeanes et al., 2008). For instance, exogenous overexpression of cadherins inhibits Wnt/β-catenin signlaing in *Xenopus*, *Drosophila* and other cellular systems (Fagotto et al., 1996;Heasman et al., 1994;Orsulic et al., 1999;Sanson et al., 1996). Thus, these data suggest that the loss of the tumor suppressor E-cadherin promotes tumorigenesis by stimulating the Wnt/β-catenin signaling pathway.

1.2.2.2 Modulation of growth factor signaling

E-cadherin may also control tumor development via its influence on mitogenic signaling. For example, in the lung carcinoma cell line PC9, restoration of the E-cadherin-catenin adhesion system resulted in cell-cell cohesion and also caused a retardation of cell proliferation, suggesting that functional E-cadherin adhesion might be involved in contact-dependent growth inhibition (Watabe et al., 1994). Furthermore, it has been demonstrated that E-cadherin inhibits

or modulates signaling through the epidermal growth factor receptor (EGFR). First, E-cadherin can physically interact with the EGFR and also with the ErbB RTK family (Fedor-Chaiken et al., 2003;Hoschuetzky et al., 1994;Ochiai et al., 1994;Qian et al., 2004). Second, cells expressing E-cadherin have less responsiveness to EGF activation (Perrais et al., 2007;Qian et al., 2004). It is likely that E-cadherin inactivates EGFR signaling via direct interaction with EGFR, thereby altering the ligand-binding site within EGFR, which results in the inability of EGF to stimulate EGFR (Qian et al., 2004).

1.2.2.3 Signaling through Rho GTPases

Another potential mechanism induced by the E-cadherin dysfunction during tumor progression may include the rearrangement of actin cytoskeleton. Rho family of small GTPases, which includes RhoA, Cdc42 and Rac1, plays a key functional role in regulating cytoskeleton, cell adhesion, and cell motility (Bourne et al., 1991). Rho GTPases have also been shown to be involved in tumor cell proliferation, survival, transformation, and progression to malignancy (Sahai and Marshall, 2002). As with other small GTPases, the members of this family shift between an inactive GDP-bound and an active GTP-bound conformation promoted by guanine nucleotide exchange factors (GEFs) whereas GTPase activating proteins (GAPs) inactivates Rho GTPases. Cdc42 triggers membrane projections (filopodia); while Rac1 is responsible for the formation of membrane ruffles (lamellipodia) at the leading edge of migrating cells; and RhoA modulates stress fiber and focal adhesions. Moreover, Cdc42 is crucial for defining the direction of migration, and Rac1 is required for forward cellular movement (Raftopoulou and Hall, 2004a;Ridley et al., 2003a).

Numerous studies have demonstrated that E-cadherin homophilic ligation can stimulate Rac1 activation in Madin-Darby Canine Kidney Epithelial (MDCK) cells (Fukuyama et al., 2006;Kovacs et al., 2002;Nakagawa et al., 2001;Perez et al., 2008). E-cadherin homophilic adhesion can also activate Rac1 by recruiting PI3K to the cadherin complex (Cavallaro and Christofori, 2004;Nakagawa et al., 2001). By contrast, overexpression of E-cadherin in E-cadherin-deficient MDA-MB-231 cells markedly reduced Rac1 activity. Furthermore, downregulation of E-cadherin using shRNA elevates Rac1 activity in A431 (squamous), Caco-2 (intestinal epithelial), and MCF7 (breast) cells (Soto et al., 2008), suggesting that E-cadherin regulates Rac1 activity depending on cellular context. There is now emerging evidence that p120-catenin also is involved in the loss of E-cadherin-induced tumor invasion and metastasis (Yanagisawa and Anastasiadis, 2006). p120-catenin is important for these processes by either stabilizing E-cadherin or by promoting cell migration and invasion in E-cadherin-deficient cells by regulating of Rho GTPase activities (Yanagisawa et al., 2008;Yanagisawa and Anastasiadis, 2006). It has been found that p120-catenin regulates the activity of Rho GTPase in a cadherin/adhesion-dependent manner (Anastasiadis, 2007). p120-catenin suppresses RhoA activity by recruiting the Rho inhibitor p190Rho GAP to the cadherin complexes (Wildenberg et al., 2006) or by inhibiting GDP dissociation and therefore activation of RhoA in the E-cadherin-unbound state (Anastasiadis et al., 2000;Castano et al., 2007;Yanagisawa et al., 2008). Moreover, overexpression of p120-catenin stimulates Rac1 activity in fibroblasts (Grosheva et al., 2001;Noren et al., 2000), whereas E-cadherin activates Rac1 in the cadherin/adhesion-dependent manner via binding of p120-catenin to the cadherin complexes (Gavard et al., 2004;Goodwin et al., 2003). However, it remains to be determined how E-cadherin modulates Rho GTPase activity during tumour progression.

1.2.3 The roles of E-cadherin in ovarian cancer progression

In the human ovary, E-cadherin is not expressed in OSE cells, but is expressed in the epithelium of cortical inclusion cysts (Sundfeldt et al., 1997). *In vitro* experiments have shown that overexpression of E-cadherin in human immortalized OSE cells initiated adherens junction formation, and regulates OSE cell morphology and migration (Wu et al., 2008a). However, loss of E-cadherin has been described in borderline and malignant ovarian tumors, when compared with benign tumors (Daraï et al., 1997), and has been associated with high tumor grade, peritoneal dissemination and poor prognosis (Cho et al., 2006;Daraï et al., 1997). In addition, ascites cells in ovarian cancer with low E-cadherin expression were more invasive than the corresponding solid tumor cells (Veatch et al., 1994). Consistent with this notion, forced overexpression of E-cadherin in the tumorigenic E-cadherin-deficient cell line, RERF-LCAI, inhibited ovarian metastasis in the xenograft model (Kuwabara et al., 2008). Moreover, an *in vivo* model demonstrated that knockdown expression of E-cadherin by siRNA in ovarian cancer cells promoted cancer metastasis via integrin α5 (Sawada et al., 2008).

Many growth factors [e.g. transforming growth factor β (TGFβ), EGF, hepatocyte growth factor (HGF) and endothelin 1 (ET1)] have been shown to down-regulate E-cadherin expression in ovarian cancer (Vergara et al., 2010). However, the underlying molecular mechanism by which these growth factors suppress E-cadherin expression remains unclear.

1.3 Hypothesis and objectives:

<u>The hypothesis of this study is:</u>

Loss of E-cadherin function contributes to the growth factor-induced tumor growth and invasion in ovarian cancer.

The specific objectives of this work are:

Objective 1. To delineate the effect of FGF2 on ovarian cancer cell invasion. (Chapter 2)

1) To determine the effect of FGF2 on E-cadherin expression in ovarian cancer cells.

2) To define the downstream molecules (i.e. Slug and ZEB1) involved in FGF2-induced E-cadherin down-regulation.

3) To study the role of FGF2 in ovarian cancer cell invasiveness.

4) To determine the downstream signaling pathways (i.e., MAPK/ERK and PI3K/Akt/mTOR) involved in FGF2-induced E-cadherin down-regulation, and the cell invasiveness.

Objective 2. To examine the effect of IGF1 on ovarian cancer cell proliferation. (Chapter 3)

1) To determine the effect of IGF1 on E-cadherin expression in ovarian cancer cells.

2) To define downstream molecules (i.e. Snail and Slug) involved in IGF1-induced E-cadherin down-regulation.

3) To study the role of IGF1 in ovarian cancer cell proliferation.

4) To demonstrate the downstream signaling pathways (i.e., PI3K/Akt/mTOR) involved in IGF1-induced E-cadherin down-regulation, and the cell proliferation.

Objective 3. To elucidate the role of E-cadherin in ovarian cancer cell growth. (Chapter 4)

1) To study the effect of E-cadherin in ovarian cancer cell growth.

2) To determine the downstream signaling pathways (i.e., PI3K/Akt) involved in the loss of E-cadherin-enhanced the cell proliferation.

3) To define the molecular mechanisms (i.e. β-catenin-dependent PTEN down-regulation) by which the loss of E-cadherin-induced the activation of the PI3K/Akt signaling pathway.

Objective 4. To elucidate the role of E-cadherin in ovarian cancer cell invasion. (Appendix 1)

1) To demonstrate the effect of E-cadherin in ovarian cancer cell invasiveness.

2) To identify the downstream signaling pathways (i.e., PI3K/Akt) involved in the loss of E-cadherin-enhanced the cell invasiveness.

2. Fibroblast growth factor 2 induces E-cadherin down-regulation via PI3K/Akt/mTOR and MAPK/ERK signaling in ovarian cancer cells

2.1 Introduction

Epithelial ovarian cancer (EOC), which comprises 90% of all ovarian malignancies, is the most common and lethal form of gynecological cancer in developed countries (Jemal et al., 2005), the death rate for this disease has not changed much in the last 50 years.

Fibroblast growth factor 2 (FGF2) mediates various cellular functions, including proliferation, motility, and differentiation (Bottcher and Niehrs, 2005;Chalkiadaki et al., 2009), and malignant ovarian tumors are common in patients with elevated FGF2 (Barton et al., 1997;Davidson et al., 2002;Fujimoto et al., 1997). However, the role of FGF2 in ovarian cancer progression is still controversial. Ovarian tumors with high cytoplasmic FGF2 are associated with reduced tumor aggressiveness and increased survival rates compared with patients with low levels of FGF2 (Obermair et al., 1998;Secord et al., 2007). In contrast, previous *in vitro* studies and the gene expression profiling studies in advanced ovarian cancer implicate that FGF2 functions as an autocrine growth factor for ovarian cancer cell proliferation (Crickard et al., 1994;De Cecco et al., 2004;Di Blasio et al., 1993) and invasion (Li and Jiang, 2010). Moreover, FGF2 regulates the expression of additional genes implicated in angiogenesis or metastasis, including metalloproteinases (Strutz et al., 2002), vascular endothelial growth factor (Giavazzi et al., 2003), and E-cadherin (Billottet et al., 2004;Strutz et al., 2002;Wu et al., 2008b).

E-cadherin functions as a cell-cell adhesion molecule and tumor suppressor that is silenced in various cancers, and the loss of E-cadherin function or expression is a common event in tumor progression (Nollet et al., 1999;Yap, 1998). E-cadherin is known to suppress tumor cell invasion, and the re-expression of E-cadherin in E-cadherin-deficient carcinomas results in a less

invasive, less aggressive behavior (Gottardi et al., 2001;St Croix et al., 1998;Yanagisawa and Anastasiadis, 2006), while the loss of E-cadherin is correlated with ovarian cancer metastasis, peritoneal dissemination, and poor prognosis (Daraï et al., 1997;Hudson et al., 2008;Sawada et al., 2008;Veatch et al., 1994;Yuecheng et al., 2006). The loss of E-cadherin function can be achieved by the mutation of the E-cadherin gene (Hajra and Fearon, 2002), the hypermethylation of the E-cadherin promoter (Graff et al., 1995;Hennig et al., 1995), and the transcriptional repression of E-cadherin (Batlle et al., 2000;Cano et al., 2000;Comijn et al., 2001;Hajra et al., 2002). Several transcription factors have been identified to suppress E-cadherin including Snail, Slug, Twist and ZEB1 via their interaction with the E-box binding site in the E-cadherin promoter (Batlle et al., 2000;Bolos et al., 2003;Cano et al., 2000;Grooteclaes and Frisch, 2000;Peinado et al., 2007).

Previous studies have demonstrated that FGF2 suppresses E-cadherin in various cell types (Billottet et al., 2004;Strutz et al., 2002;Wu et al., 2008b); however, the underlying mechanisms are still largely unknown. Here, we demonstrate that FGF2 reduces E-cadherin mRNA and protein levels in a time- and dose-dependent manner. Furthermore, increased Slug and ZEB1 expression via the activation of the PI3K/Akt/mTOR and the MAPK/ERK signaling pathways, respectively, potentially mediates the effects of FGF2 on E-cadherin. Finally, our results indicate that the down-regulation of E-cadherin mediates FGF2-enhanced the invasiveness in ovarian cancer cells.

2.2 Materials and methods

2.2.1 Materials

FGF2 was purchased from Sigma-Aldrich (Ontario, Canada). Rapamycin, U0126 and

wortmannin were purchased from Calbiochem (San Diego, CA). E-cadherin antibodies were purchased from BD Biosciences (San Jose, CA). Akt, phospho-Akt (Ser473), p44/42 MAPK (ERK), phospho-p44/42 MAPK (Thr202/Tyr204), p70S6K and phospho-p70S6K (Thr389) antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA). The β-actin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG antibodies were purchased from Bio-Rad Laboratories (Hercules, CA).

2.2.2 Plasmid constructs

The pcDNA-GFP (GFP) and were generously provided by Dr. Alonzo H. Ross (Liu et al., 2005). The pcDNA-Ecadherin-GFP (Ecad-GFP) was a kind gift from Dr. Jennifer L. Stow (Miranda et al., 2001).

2.2.3 Cell culture and transfections

Human ovarian cancer cell lines (OVCAR-4 and SKOV-3) were purchased from the American Type Culture Collection (ATCC, Manassas, VA), and their use was approved by the University of British Columbia Clinical Screening Committee for Research and Other Studies Involving Human Subjects. Cells were cultured in Medium 199:MCDB 105 (1:1; Sigma-Aldrich) containing 10 % fetal bovine serum (FBS; Hyclone Laboratories Ltd., Logan, UT), 100 U/ml penicillin G and 100 μg/ml streptomycin (Life Technologies, Inc., Rockville, MD) in a humidified atmosphere of 5 % CO_2 to 95 % air at 37°C. The cells were passaged with 0.06 % trypsin (1:250)/ 0.01 % EDTA in Mg^{2+} /Ca^{2+} - free HBSS at confluence.

All transfections were carried out using Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's protocol.

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

Total RNA was prepared using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Single-stranded cDNA was synthesized from 2 μg total RNA according to the manufacturer's procedure (Amersham Biosciences, Quebec, Canada). The primers used for SYBR Green RT-qPCR were as follows: for human E-cadherin, sense, 5'-ACA GCC CCG CCT TAT GAT T-3' and antisense, 5'-TCG GAA CCG CTT CCT TCA-3'; for Slug, sense, 5'-TTC GGA CCCACA CAT TAC CT-3' and antisense, 5'-GCA GTG AGG GCA AGA AAA AG-3' ; for ZEB1, sense, 5'-GCA CCT GAA GAG GAC CAG AG-3' and antisense, 5'-TGC ATC TGG TGT TCC ATT TT-3'; and for GAPDH, sense, 5'-ATG GAA ATC CCA TCA CCA TCT T-3' and antisense, 5'-CGC CCC ACT TGA TTT TGG -3'. RT-qPCR was performed using the Applied Biosystems 7300 Real-Time PCR System equipped with a 96-well optical reaction plate. Relative quantification of mRNA levels was performed using the comparative Cq method (ΔΔCq method) with GAPDH as the reference gene.

2.2.5 Western blot analysis

Cells were harvested in lysis buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 1 mM EDTA, 0.1% SDS] containing protease inhibitor cocktail (Sigma-Aldrich), and protein concentrations were determined using the DC Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA). Protein (40 μg) was electrophoresed on 7.5 % SDS-polyacrylamide gels, transferred to nitrocellulose membranes (Amersham Bioscience), and

incubated with specific primary antibodies at 4 ^{o}C overnight. After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h and were visualized with enhanced chemiluminescent substrate (Thermo Fisher Scientific Inc, Waltham, MA).

2.2.6 Invasion assay

Twenty-four-well transwell inserts with an 8-µm pore coated with 1mg/ml Matrigel (50 µl/well; BD sciences, Mississauga, ON, Canada) were used to assess cell invasion. Trypsinized cells (1×10^5) in 0.1% FBS medium, with or without FGF2, were seeded in triplicate in the upper chamber. 1% FBS medium was placed in the lower wells. The chambers were incubated for 24 h at 37^{o}C in a 5% CO_2 atmosphere. Cells that did not penetrated the filter were wiped off, and invaded cells on the lower surface of the filter were fixed with ice-cold methanol and stained with 0.5% crystal violet. Results are presented as the mean number of invaded cells of five fields (at 100x magnification) ± SEM from three independent experiments.

2.2.7 Data analysis

All values are expressed as mean ± SEM from three to six independent experiments. Data were analyzed by a one-way ANOVA followed by Tukey's *post hoc* test using GraphPad Prism 5 (GraphPad Software, San Diego, CA). P <0.05 was considered statistically significant.

2.3 Results

2.3.1 Effect of FGF2 on E-cadherin expression in ovarian cancer cells

As a first step toward analyzing the role of FGF2 in ovarian cancer progression, I investigated the effect of FGF2 on E-cadherin expression in OVCAR-4 and SKOV-3 cells. Our results showed that treatment with FGF2 down-regulated E-cadherin mRNA levels in both a time- (Figure 2.1A) and dose-dependent manner (Figure 2.1B). Similarly, Western blot analysis showed that treatment with FGF2 down-regulated E-cadherin protein levels in a dose-dependent manner in ovarian cancer cells (Figure 2.1C).

2.3.2 FGF2-induces E-cadherin down-regulation via the PI3K/Akt and MAPK/ERK pathways

It is well documented that the PI3K/Akt and MAPK/ERK pathways are frequently amplified and serve as survival pathways in ovarian carcinomas (Nicosia et al., 2003). In addition, FGF2 is known to activate the PI3K/Akt and MAPK/ERK pathways (Eswarakumar et al., 2005;Schlessinger, 2004) and, has been reported to regulate E-cadherin down-regulation (Graham et al., 2008;Saegusa et al., 2009). Therefore, we analyzed whether these two pathways were involved in the suppression of E-cadherin expression by FGF2. We first examined the phosphorylation status of Akt and ERK upon treatment with 10 ng/ ml FGF2 at 5, 15, 30, 60, and 120 minutes post-treatment and found that FGF2 treatment induced the phosphorylation of Akt (Ser473) and ERK (Thr202/Tyr204) in a time-dependent manner in SKOV-3 cells (Figure 2.2A). To determine whether these two pathways were involved in the suppression of E-cadherin expression by FGF2, we used two pharmacological inhibitors, wortmannin and U0126, to specifically block the PI3K/Akt and MAPK/ERK pathways in SKOV-3 cells (Figure 2.2B), respectively. As shown in Fig. 2C, the PI3K and ERK inhibitors significantly increased basal E-

cadherin expression and markedly diminished, but did not completely abolish, FGF2-induced E-cadherin expression, which demonstrated the involvement of the PI3K/Akt and MAPK/ERK pathways in the FGF2-mediated down-regulation of E-cadherin expression in ovarian cancer cells.

2.3.3 FGF2 deferentially up-regulates Slug and ZEB1expression via the PI3K/Akt and MAPK/ERK pathways, respectively

To determine if FGF2 induced E-cadherin down-regulation by regulating the transcription of E-cadherin, the mRNA levels of the E-cadherin transcriptional repressors Snail, Slug, Twist and ZEB1 were examined by RT-qPCR. Treatment with FGF2 significantly elevated Slug and ZEB1 mRNA levels in a time- (Figure 2.3A) and dose-dependent manner (Figure 2.3B) but had no significant influence on Snail and Twist mRNA levels (data not shown). To investigate if the PI3K/Akt and MAPK/ERK signaling pathways are involved in FGF2-induced increases in Slug and ZEB1 mRNA, cells were treated with PI3K inhibitor (wortmannin) or MEK inhibitor (U0126) in the presence or absence of FGF2. Interestingly, wortmannin significantly suppressed the basal Slug mRNA level and totally abolished the effects of FGF2 on Slug mRNA levels (Figure 2.3C), whereas FGF2-enhanced ZEB1 mRNA levels were not affected (Figure 2.3D). On the other hand, U0126 treatment totally abolished the effects of FGF2 on ZEB1 mRNA levels (Figure 2.3D), the drug had no effect on Slug mRNA levels (Figure 2.3C).

2.3.4 FGF2-induced E-cadherin down-regulation via the PI3K/Akt/mTOR pathway

Next, we investigated the role of the mTOR pathway in FGF2-induced E-cadherin down-

regulation, because mTOR is a pathway downstream of PI3K/Akt signaling that has been shown to be involved in E-cadherin down-regulation (van der Poel, 2004). As shown, treatment with FGF2 induced the activation of mTOR signaling in a time-dependent manner in SKOV-3 cells, as indicated by phosphorylation of the mTOR downstream molecule, p70S6K (Figure 2.4A). To determine whether the PI3K/Akt/mTOR signaling pathway was involved in the regulation of E-cadherin levels by FGF2, we used the mTOR-specific inhibitor rapamycin to block the mTOR pathway in SKOV-3 cells (Figure 2.4B). Similar to wortmannin, rapamycin totally abolished the FGF2-induced elevation of Slug mRNA (Figure 2.4C), whereas rapamycin showed no effect on ZEB1 mRNA levels (Figure 2.4D). Moreover, rapamycin treatment significantly elevated the protein levels of E-cadherin and markedly diminished the suppressive effect of FGF2 on E-cadherin protein levels (Figure 2.4E), indicating that the PI3K/Akt/mTOR pathway is involved in FGF2-induced E-cadherin suppression in ovarian cancer cells.

2.3.5 Activation of the PI3K/Akt/mTOR and MAPK/ERK signaling pathways are critical for FGF2-induced cell invasion

Several lines of evidence indicate that FGF2 plays a key role in the invasive properties of human cancer cells (Blair et al., 2010;Kamura et al., 2010). Thus, we examined the effect of FGF2 on cell invasion in ovarian cancer cells using Matrigel-coated Transwell invasion assays. OVCAR-4 and SKOV-3 cells were treated with increasing concentrations of FGF2, resulted in a dose-dependent stimulation of invasion (Figure 2.5A). The involvement of the PI3K/Akt/mTOR and MAPK/ERK signaling pathways in FGF2-stimulated cell invasion were also evaluated. Our results showed that FGF2-induced cell invasion was markedly diminished, but not totally, by treatment with wortmannin, mTORrapamycin or U0126 (Figure 2.5B). Taken together, these

results indicate that the ERK and PI3K/Akt/mTOR pathways are involved in FGF2-induced ovarian cancer cell invasion.

2.3.6 Down-regulation of E-cadherin mediates FGF2-stimulated ovarian cancer cell invasion

Next, we asked whether down-regulation of E-cadherin mediate FGF2-induced cell invasion. SKOV-3 cells were transiently transfected with wild-type human E-cadherin expression plasmid for 48h and then were treated with FGF2 for further 24 h (Figure 2.6A). FGF2 treatment reduced E-cadherin protein levels in cells transfected with empty vector, whereas no effect was detected in cells overexpressing E-cadherin (Figure 2.6A). Overexpression of E-cadherin decreased basal invasiveness and abolished the ability of FGF2 to induce ovarian cancer cell invasion (Figure 2.6B), implicating that E-cadherin plays an essential role in FGF-stimulated ovarian cancer cell invasion.

2.4 Discussion

FGF2 normally presents in plasma at a concentration less than 10 pg/ml, and elevated levels (up to 6 ng/ml) can be found in ascetic fluid from ovarian cancer patients (Barton et al., 1997). The elevated levels of FGF2 and its receptors present in ovarian malignant tumors suggest that FGF2 plays a significant role in ovarian tumor progression (Crickard et al., 1994). However, the role of FGF2 in ovarian tumor progression remains to be elucidated. The present study shows that FGF2 induced the down-regulation of E-cadherin expression, which is involved in FGF2-induced ovarian cancer cell invasion. In addition, our studies suggest that FGF2 exerts its effects in human ovarian cancer cells via the activation of the PI3K/Akt/mTOR and MAPK/ERK signaling pathways and the subsequent increased expression of Slug and ZEB1.

FGF2 has been reported to modulate E-cadherin expression in a variety of cell types. However, its role on E-cadherin expression seems to be cell type specific. In pancreatic adenocarcinoma, FGF1 and FGF2 has been shown to up-regulate E-cadherin expression (El-Hariry et al., 2001). In contrast, the down-regulation of E-cadherin expression with FGF2 treatment has been observed in tubular epithelial cells and NBT-II carcinoma cells, resulting in increased cell migration and invasion (Billottet et al., 2004;Strutz et al., 2002). Also, FGF2 has been shown to down-regulate E-cadherin expression in human umbilical vein endothelial cells via the JNK signaling pathway (Wu et al., 2008b). The current data support a crucial role for FGF2 in the down-regulation of E-cadherin in ovarian carcinoma cells via the PI3K/Akt/mTOR and MAPK/ERK signaling pathways. The binding of FGF2 to its receptors leads to the activation of downstream signaling pathways such as PI3K/Akt and MAPK/ERK (Eswarakumar et al., 2005;Schlessinger, 2004). Emerging evidence suggests that these pathways are involved in the regulation of E-cadherin (Graham et al., 2008;Saegusa et al., 2009). Moreover, the aberrant inhibition of the mTOR pathway, which is downstream of PI3K/Akt signaling blocked FGF2-induced E-cadherin down-regulation and cell invasion. These results are consistent with a previous study demonstrating a requirement for mTOR signaling in E-cadherin down-regulation in prostate cancer cells (van der Poel, 2004). Taken together, our results indicate that the FGF2-dependent PI3K/Akt/mTOR and MAPK/ERK activation is involved in FGF2-induced E-cadherin down-regulation and cell invasion in ovarian cancer cells.

The loss of E-cadherin gene expression is mainly due to an overexpression of transcriptional repressors including Snail, Slug and ZEB1 (Bolos et al., 2003;Grooteclaes and Frisch, 2000;Peinado et al., 2007). Indeed, elevated Slug and ZEB1 mRNA levels have been found in ovarian carcinoma (Elloul et al., 2006;Elloul et al., 2010). Moreover, a previous study

demonstrated that the overexpression of Slug in SKOV-3 cells results in the down-regulation of E-cadherin, enhanced motility and invasiveness (Kurrey et al., 2005). However, much less is known about the regulation of these transcriptional repressors. Slug expression can be regulated by PI3K/Akt signaling, which can also be activated by FGF treatment (Blair et al., 2010;Eswarakumar et al., 2005;Saegusa et al., 2009;Schlessinger, 2004). Consistent with these results, the inhibition of PI3K/Akt signaling by wortmannin reduced the basal and abolished FGF2-induced Slug levels expression suggesting that this pathway is critical for Slug expression. The activation of PI3K/Akt signaling has been demonstrated to stimulate Slug expression via GSK-3β/β-catenin signaling and to subsequently down-regulate E-cadherin in uterine carcinosarcomas and normal hepatocytes (Saegusa et al., 2009;Wu et al., 2011). It is well known that PI3K/Akt signaling induces nuclear β-catenin accumulation through the inhibition of GSK3β (Cross et al., 1995;Li and Sun, 1998). In addition, the inhibition of mTOR signaling by rapamycin blocks increased β-catenin translocation into the nucleus in human pancreatic β-cells (Liu et al., 2009). FGF2 may activate PI3K/Akt signaling and its downstream GSK3β and mTOR pathways to induce β-catenin-dependent transcription including that of Slug. Further studies are required to elucidate the precise mechanism of the elevation of Slug levels by the mTOR pathway. Notably, E-cadherin protein levels were clearly increased by rapamycin, while basal Slug mRNA levels were not affected. Thus, our results suggest that the mTOR signaling pathway also modulates the E-cadherin levels in Slug-independent manner. Interestingly, treatment with the MEK inhibitor U0126 only blocked FGF2-induced ZEB1 expression, but did not inhibit FGF2-induced Slug expression. Our data suggest that MAPK/ERK is an upstream factor of ZEB1 activation in ovarian cancer cells *in vitro*. FGF2 has been shown to activate the MAPK/ERK pathway in various cancer cells (Hatziapostolou et al., 2006;Wesley et al., 2005),

and we show in OVCAR-4 and SKOV-3 cells that ZEB1 expression is MAPK/ERK-dependent. These results are consistent with a previous study demonstrating a requirement for MAPK/ERK signaling in IGF-1-induced ZEB1 expression in prostate cancer cells (Graham et al., 2008). Furthermore, a recent study demonstrated that ERK2, but not ERK1, reduced E-cadherin levels via Fra1-mediated ZEB1/2 in a human mammary epithelial cell line, MCF-10A cells (Shin et al., 2010). Taken together, our findings indicate that FGF2 deferentially regulates Slug and ZEB1 expression via the PI3K/Akt/mTOR and MAPK/ERK signaling pathways in human ovarian cancer cells.

The biological significance of E-cadherin reduction in ovarian cancer invasiveness was demonstrated by the fact that overexpression of E-cadherin blocked FGF2-induced cell invasion *in vitro*. Evidence indicates that the loss of E-cadherin is associated with ovarian cancer metastasis, peritoneal dissemination and poor patient survival (Daraï et al., 1997;Hudson et al., 2008;Sawada et al., 2008;Veatch et al., 1994;Yuecheng et al., 2006), suggesting that E-cadherin functions as a suppressor of tumor invasiveness. Indeed, silencing E-cadherin by siRNA enhances ovarian cancer cell invasion via an up-regulation of the α5-integrin (Sawada et al., 2008). We have also recently found that E-cadherin knockdown by RNA interference increases the PI3K/Akt signaling pathway (Lau et al., 2011), which mediates E-cadherin-depletion-induced invasion in ovarian cancer cells (Lau *et al*., unpublished). Moreover, the overexpression of a dominant-negative E-cadherin mutant in ovarian carcinoma cells results in increased mesenchymal cell migration (Wu et al., 2008a). The present results demonstrate that FGF2 enhances cell invasiveness by down-regulating E-cadherin and that E-cadherin overexpression inhibits basal invasiveness and abolishes FGF2-induced invasion. Previous studies demonstrated that the PI3K/Akt and MAPK/ERK signaling pathways are involved in FGF2-induced cell

invasion (Blair et al., 2010;Kamura et al., 2010). Furthermore, additional mechanisms such as the elevation of protease activity/secretion, the modulation of actin cytoskeleton, and increased motility have also been described to mediate FGF2-induced cell invasion (Kamura et al., 2010;Lee and Kay, 2006;Li and Jiang, 2010). Taken together, these results demonstrate that E-cadherin acts as a crucial suppressor of ovarian cancer invasiveness, and along with other described mechanisms, the loss of E-cadherin plays an important role in FGF2-induced cell invasion.

In summary, our results show that FGF2 down-regulates E-cadherin expression, most likely through the transcriptional suppression of Slug and ZEB1, which are concomitantly expressed by the activation of the PI3K/Akt/mTOR and MAPK/ERK pathways. In addition, the present study suggests that the down-regulation of E-cadherin mediates FGF2-induced ovarian cancer cell invasion (Figure 2.7). Inhibition of either PI3K/Akt/mTOR or MAPK/ERK signaling results in partially blocked the FGF2-induced E-cadherin down-regulation and cell invasion. Thus, these findings indicate that the design of combined treatments targeting FGF2-related signaling cascades may have relevant implications in the treatment of this malignancy.

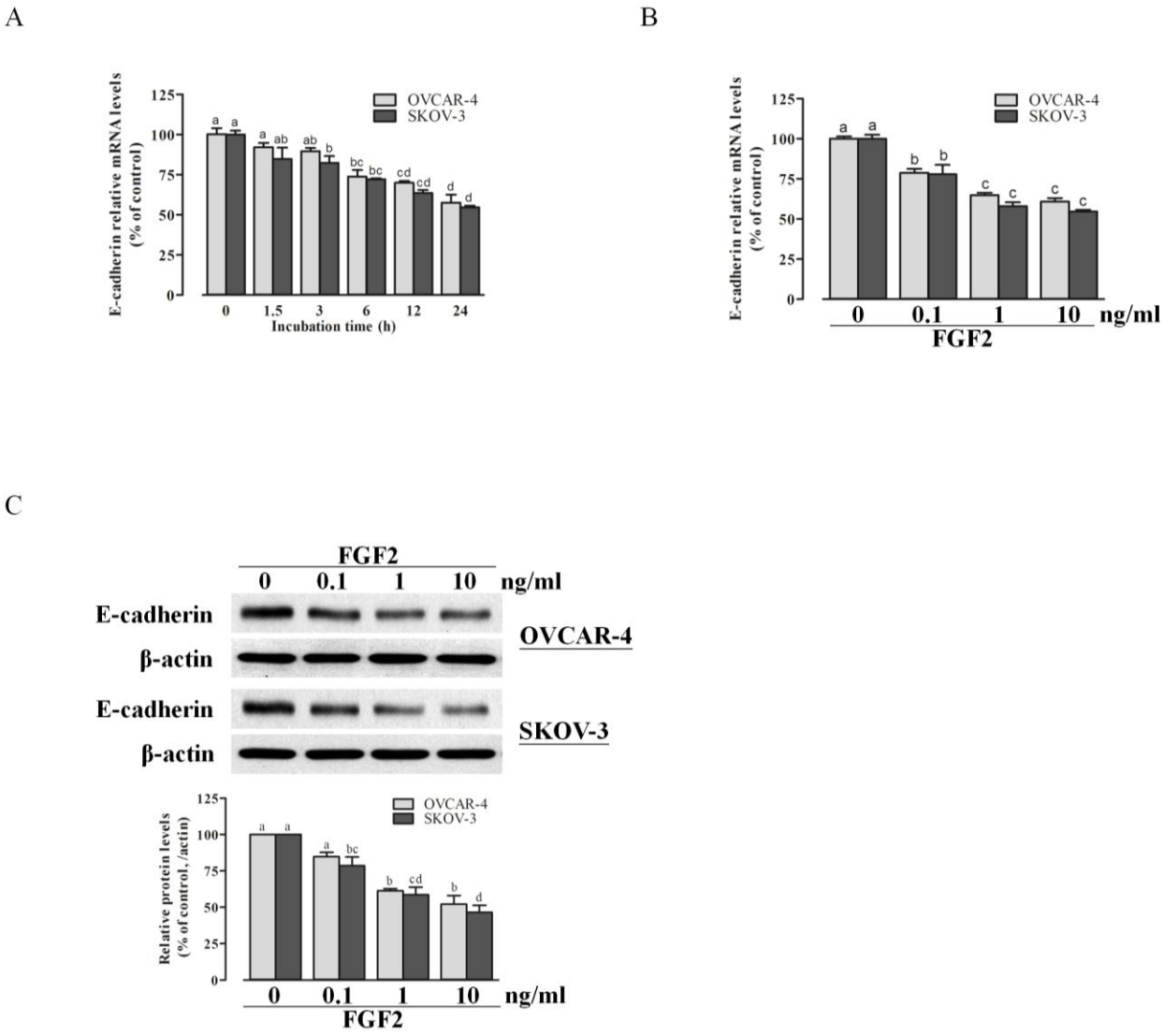


Figure 2.1. FGF2 suppresses E-cadherin mRNA and protein levels in OVCAR-4 and SKOV-3 cells. (A) OVCAR-4 and SKOV-3 cells were treated with 10 ng/ml FGF2 for 0 to 24 h as indicated, and then E-cadherin mRNA levels were analyzed by RT-qPCR. (B and C) OVCAR-4 and SKOV-3 cells were treated with different dose of FGF2 for 24 h after which E-cadherin mRNA levels (B) and protein levels (C) were analyzed by RT-qPCR and Western blotting, respectively. Results represent the mean ± SEM (n=3; values without a common letter are significantly different, $P < 0.05$). Data were analyzed by one-way ANOVA followed by Tukey's multiple comparison test.

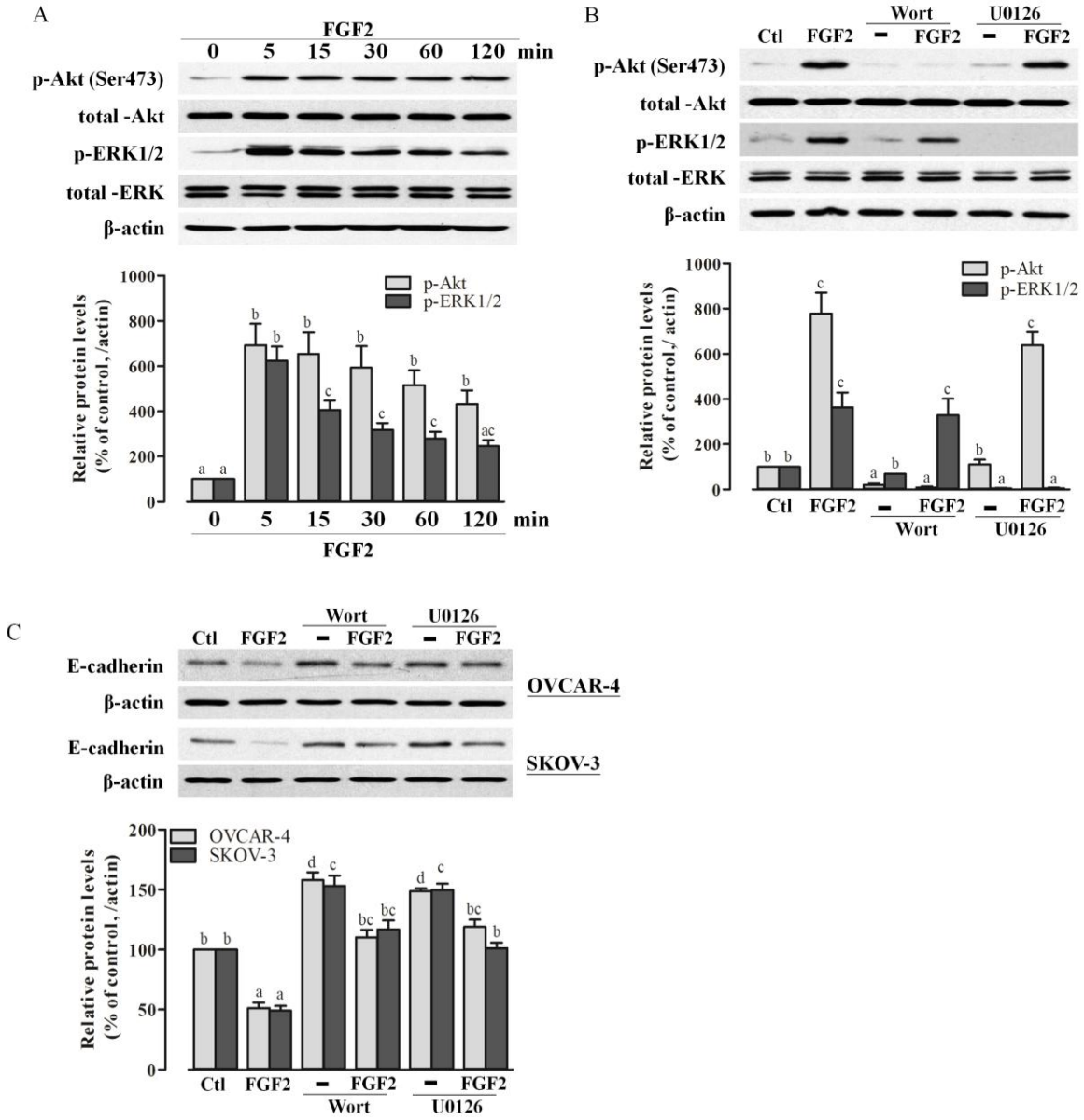


Figure 2.2. FGF2 suppresses E-cadherin expression via the PI3K/Akt and MAPK/ERK signaling pathways. (A) SKOV-3 cells were treated with 10 ng/ml FGF2 for 0 to 120 min as indicated. Phosphorylated and total Akt, phosphorylated and total ERK, and β-actin levels were analyzed by Western blot analysis. (B) SKOV-3 cells were pretreated with wortmannin (1 μM) or U0126 (10 μM) for 30 min before the addition of FGF2 (10 ng/ml) for 30 min. Phosphorylated and total Akt, phosphorylated and total ERK protein levels were analyzed by Western blotting. The β-actin antibody was used as a control for equal loading. (C) OVCAR-4 and SKOV-3 cells were pretreated with wortmannin (1 μM) or U0126 (10 μM) alone or in the presence of 10 ng/ml FGF2 for 24 h, after which E-cadherin protein levels were analyzed by Western blotting. Results represent the mean ± SEM [(A) & (B) n=3; (C) n=6; values without a common letter are significantly different, $P < 0.05$]. Data were analyzed by one-way ANOVA followed by Tukey's multiple comparison test.

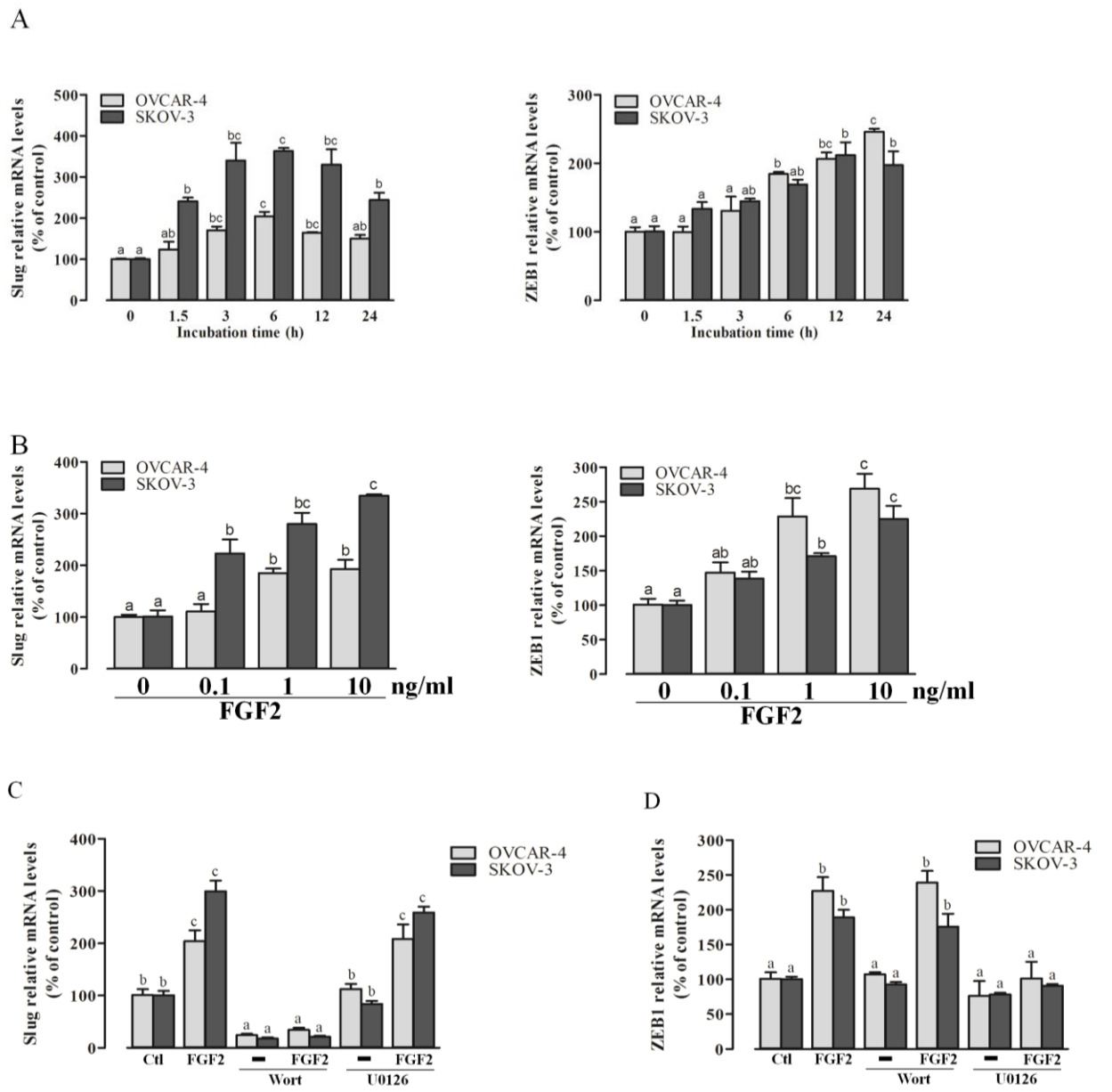


Figure 2.3. FGF2 increases Slug and ZEB1 transcriptional levels in OVCAR-4 and SKOV-3 cells. (A) OVCAR-4 and SKOV-3 cells were treated with 10 ng/ml FGF2 for various times, and the mRNA levels of Slug (*left panel*) and ZEB1 (*right panel*) were analyzed by RT-qPCR. (B) OVCAR-4 and SKOV-3 cells were treated with different doses of FGF2 for 6 h (Slug; *left panel*) or 24 h (ZEB1; *right panel*), and mRNA levels were analyzed by RT-qPCR. (C and D) OVCAR-4 and SKOV-3 cells were pretreated with wortmannin (1 μM) or U0126 (10 μM) for 30 min prior to the addition of 10 ng/ml FGF2 for 6 h (C) and 24 h (D). Slug and ZEB1 mRNA levels were analyzed by RT-qPCR. Results represent the mean ± SEM (n=3; values without a common letter are significantly different, $P < 0.05$). Data were analyzed by one-way ANOVA followed by Tukey's multiple comparison test.

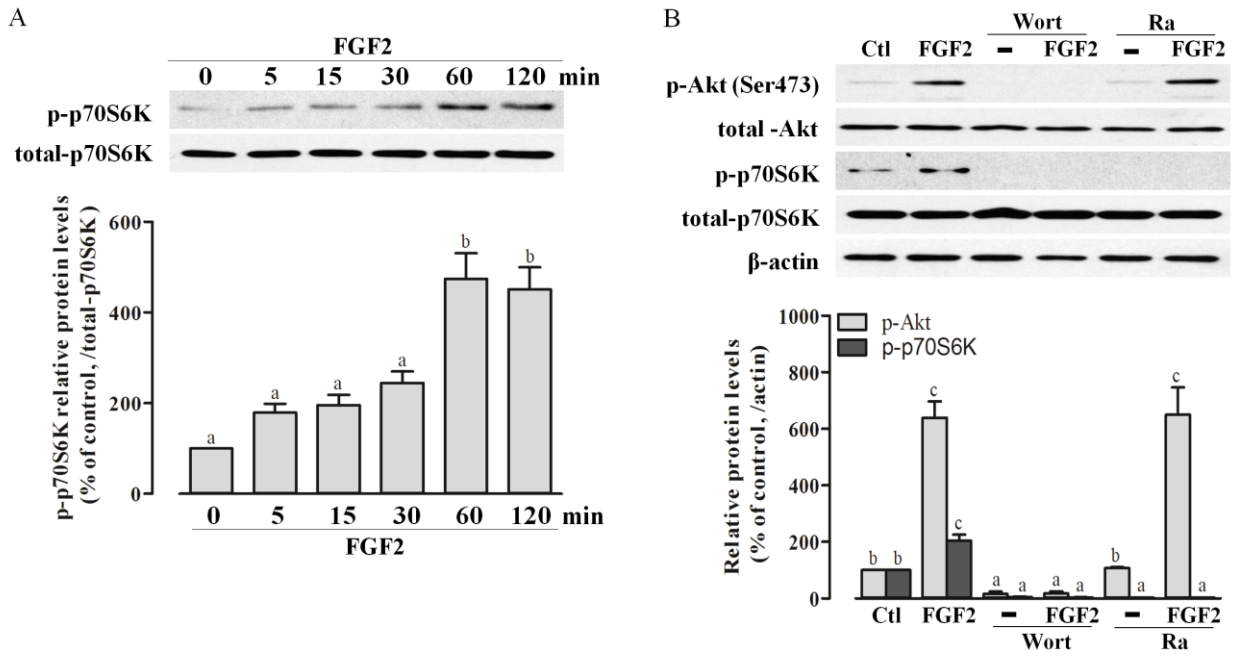


Figure 2.4. FGF2 suppresses E-cadherin expression via the PI3K/Akt/mTOR signaling pathway. (A) SKOV-3 cells were treated with 10 ng/ml FGF2 for 0 to 120 min as indicated. Phosphorylated and total p70S6K levels were analyzed by Western blot analysis. (B) SKOV-3 cells were pretreated with wortmannin (1 μM) or rapamycin (20 nM) for 30 min prior to the addition of 10 ng/ml FGF2 for 30 min. Phospho-Akt and Akt, along with phospho-p70S6K and p70S6K protein levels were analyzed by Western blotting. The β-actin antibody was used as a control for equal loading.

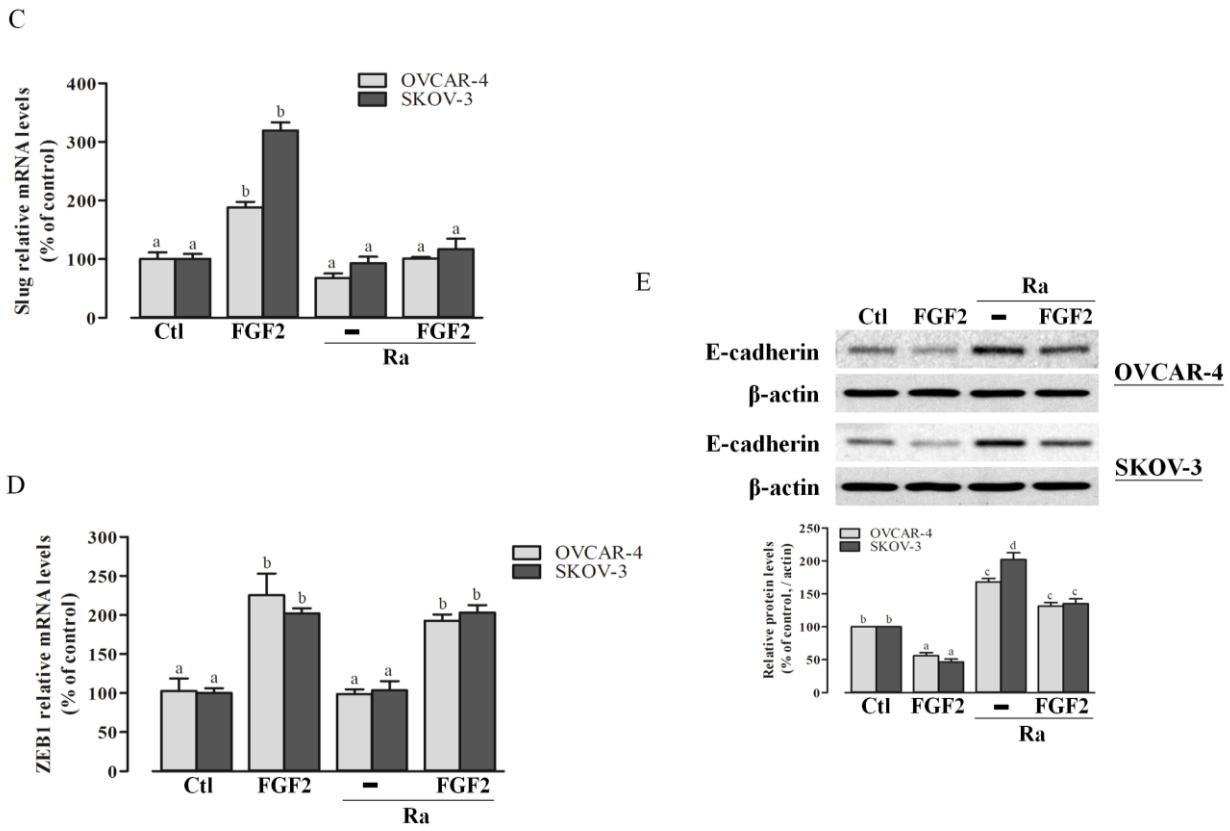
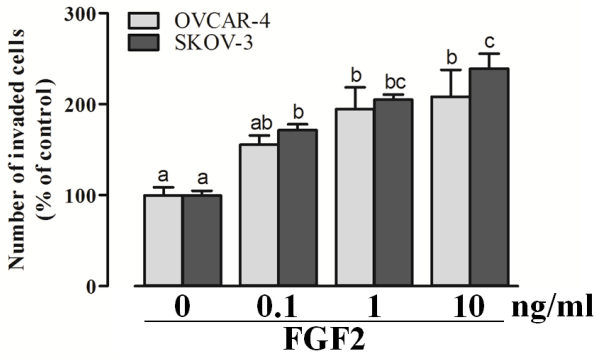


Figure 2.4. (C and D) OVCAR-4 and SKOV-3 cells were pretreated with rapamycin (20 nM) for 30 min prior to addition of 10 ng/ml FGF2 for 6 h (C) and 24 h (D). Slug and ZEB1 mRNA levels were analyzed by RT-qPCR. (E) OVCAR-4 and SKOV-3 cells were pretreated with rapamycin (20 nM) for 30 min prior to addition of 10 ng/ml FGF2 for 24 h, after which E-cadherin protein levels were analyzed by Western blotting. Results represent the mean ± SEM [(A)-(D) n=3; (E) n=6; values without a common letter are significantly different, $P < 0.05$). Data were analyzed by one-way ANOVA followed by Tukey's multiple comparison test.

A



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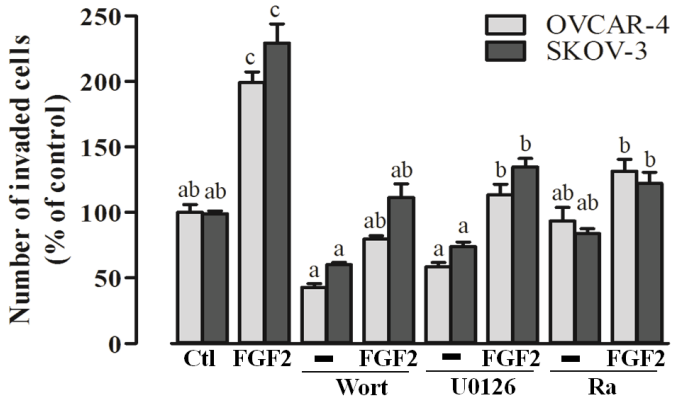
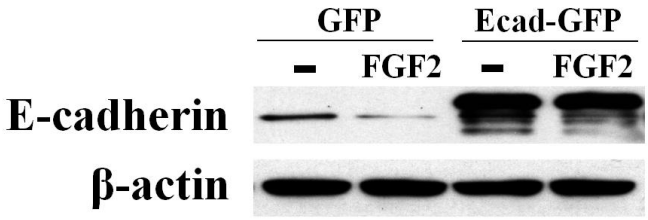


Figure 2.5. FGF2 induces ovarian cancer cell invasion via the PI3K/Akt/mTOR and MAPK/ERK signaling pathways. (A) Ovarian cancer cells were seeded in Matrigel-coated transwell inserts and treated with different doses of FGF2 for 24 h. (B) Cells were pre-treated with wortmannin (1 μM), rapamycin (20 nM) or U0126 (10 μM) for 30 min and seeded in Matrigel-coated transwell inserts and cultured with 10 ng/ml FGF2 for 24 h. Results represent the mean ± SEM [(A) n=3; (B) n=6; values without a common letter are significantly different, $P < 0.05$]. Data were analyzed by one-way ANOVA followed by Tukey's multiple comparison test.

A



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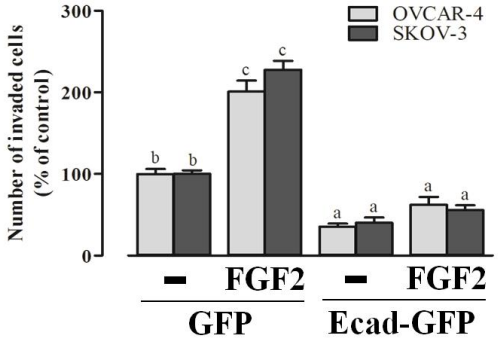


Figure 2.6. The loss of E-cadherin mediates FGF2-induced invasion. (A) SKOV-3 cells were transient transfected with the pcDNA-GFP (GFP) or human E-cadherin expression plasmids (Ecad-GFP) for 48 h. After transfection, cells were treated with 10 ng/ml FGF2 for 24 h and subjected to immunoblotting for E-cadherin and β-actin. (B) After 48 h of transfection, the trypsinized cells were seeded in Matrigel-coated transwell inserts, and cultured with 10 ng/ml FGF2 for 24 h. Results represent the mean ± SEM (n=6; values without a common letter are significantly different, $P < 0.05$). Data were analyzed by one-way ANOVA followed by Tukey's multiple comparison test.

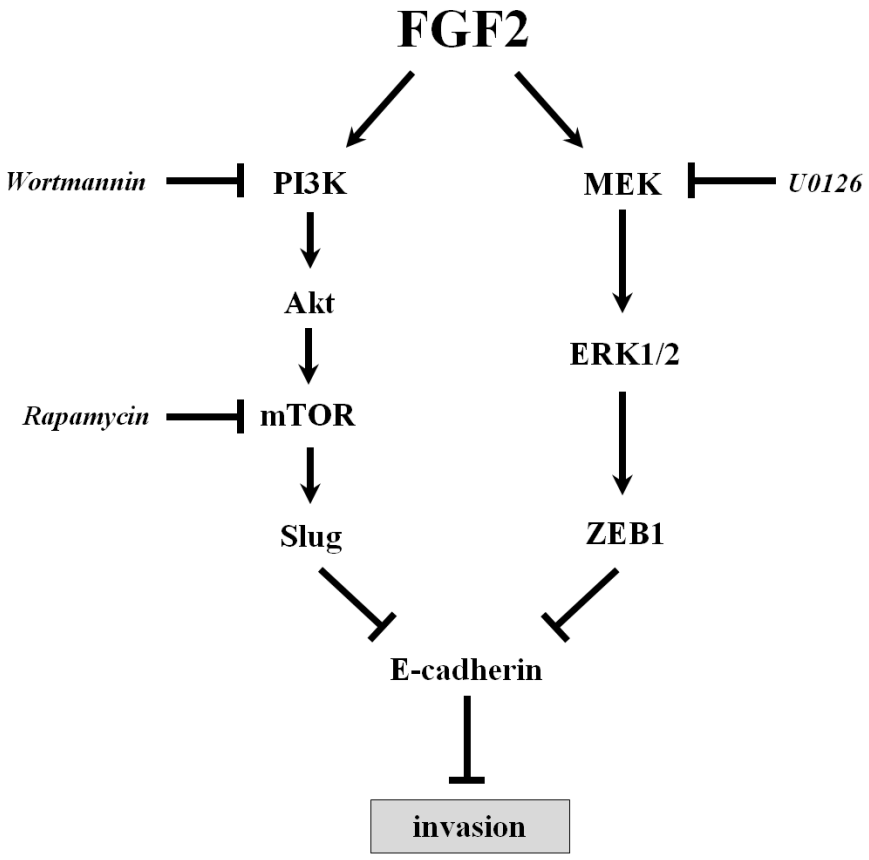


Figure 2.7. Proposed model illustrating how FGF2 suppressed E-cadherin may contribute to ovarian cancer cell invasion.

3. The PI3K/Akt/mTOR signaling pathway mediates insulin-like growth factor 1-induced E-cadherin down-regulation and cell proliferation in ovarian cancer cells

3.1 Introduction

Epithelial ovarian cancer (EOC), which comprises 90% of all ovarian malignancies, is the most lethal form of gynecological cancer among women in developed countries (Jemal et al., 2005). Due to the lack of efficient screening for the early detection and the vast majority of women are diagnosed at the advanced stage of disease, the death rate for this disease has not changed much in the last 50 years. Therefore, the identification and characterization of regulators involved in ovarian cancer progression is crucial for the development of an appropriate treatment for ovarian cancers.

Insulin-like growth factor 1 (IGF1) mediates various cellular processes, including proliferation, survival, and metabolism (Yu and Rohan, 2000). Malignant ovarian tumors are common in patients with elevated serum levels of IGF1 (Dal Maso et al., 2004;Lukanova et al., 2002). In addition, high expression levels of IGF1 in ovarian cancer are associated with an increased risk of disease, disease progression, and poor prognosis (Brokaw et al., 2007;Lukanova et al., 2002;Spentzos et al., 2007). The binding of IGF1 to IGF1 receptor (IGF1R) leads to the activation of downstream signaling pathways, such as PI3K/Akt and MAPK/ERK (Adhami et al., 2006;Pollak et al., 2004). Several model systems have demonstrated that IGF1R activation, either in relation to autocrine production of ligands by cancer cells or to higher serum levels of IGFs in the patient, promotes cancer cell proliferation and metastasis (Khandwala et al., 2000). Moreover, IGF1 regulates the expression of additional genes implicated in angiogenesis or metastasis, including matrix metalloproteinases (Saikali et al., 2008), vascular endothelial growth factor (Fukuda et al., 2002;Miele et al., 2000), and E-cadherin (Graham et al., 2008;Ivanova et

al., 2008).

E-cadherin is a calcium-dependent cell-cell adhesion molecule and functions as a tumor suppressor. The loss of E-cadherin function or expression is a common event in tumor progression (Nollet et al., 1999;Yap, 1998). The loss of E-cadherin expression is associated with ovarian cancer metastasis, peritoneal dissemination, and poor prognosis (Daraï et al., 1997;Hudson et al., 2008;Sawada et al., 2008;Veatch et al., 1994;Yuecheng et al., 2006). Many cancers are associated with both somatic and germline E-cadherin mutations (Berx et al., 1998). In addition, the loss of E-cadherin expression can be achieved by the epigenetic hypermethylation of the E-cadherin promoter (Graff et al., 1995;Hennig et al., 1995). However, in most cases, E-cadherin expression is suppressed at the transcriptional level (Batlle et al., 2000;Cano et al., 2000;Comijn et al., 2001;Hajra et al., 2002;Poser et al., 2001;Yokoyama et al., 2001). Several transcription factors have been identified to suppress E-cadherin including Snail, Slug, Twist and ZEB1 via their interaction with the E-box binding site in the E-cadherin promoter (Batlle et al., 2000;Bolos et al., 2003;Cano et al., 2000;Grooteclaes and Frisch, 2000;Peinado et al., 2007).

Previous studies have demonstrated that IGF1 suppresses E-cadherin in various cell types (Graham et al., 2008;Ivanova et al., 2008); however, the underlying mechanisms are still largely unknown. Here, we demonstrated that IGF1 reduced E-cadherin mRNA and protein levels in a time- and dose-dependent manner. Furthermore, increased Snail and Slug expression via the activation of the PI3K/Akt/mTOR signaling pathways, potentially mediated the effects of IGF1 on E-cadherin. Finally, our results indicated that the PI3K/Akt/mTOR signaling mediated IGF1-enhanced cell proliferation in ovarian cancer cells.

3.2 Materials and methods

3.2.1 Materials

IGF1 was purchased from Sigma-Aldrich (Ontario, Canada). E-cadherin antibodies were purchased from BD Biosciences (San Jose, CA). Akt, phospho-Akt (Ser473), p70S6K, phospho-p70S6K (Thr389), IGF1 receptor β, and phosphor-IGF1 receptor β (Tyr980) antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Rapamycin and wortmannin were purchased from Calbiochem (San Diego, CA). The β-actin antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), as was the horseradish peroxidase-conjugated donkey anti-goat IgG. Horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG were obtained from Bio-Rad Laboratories (Hercules, CA).

3.2.2 Cell culture

Human ovarian cancer cell lines (OVCAR-5 and SKOV-3) were purchased from the American Type Culture Collection (ATCC, Manassas, VA), and their use was approved by the University of British Columbia Clinical Screening Committee for Research and Other Studies Involving Human Subjects. Cells were cultured in Medium 199:MCDB 105 (1:1; Sigma-Aldrich, Ontario, Canada) containing 10 % fetal bovine serum (FBS; Hyclone Laboratories Ltd., Logan, UT), 100 U/ml penicillin G and 100 g/ml streptomycin (Life Technologies, Inc., Rockville, MD) in a humidified atmosphere of 5 % CO_2 to 95 % air at 37°C. The cells were passaged with 0.06 % trypsin (1:250)/ 0.01 % EDTA in Mg^{2+} /Ca^{2+} - free HBSS at confluence.

3.2.3 cDNA microarray analysis

The Oncomine 4.4 database (Compendia Bioscience, Ann Arbor, MI) and gene microarray analysis tool [http://www.oncomine.org; (Rhodes et al., 2004)] were used for data mining to explore the mRNA expression of IGF1 in ovarian tumors. The data analysis was performed using standard settings. Only studies with analysis results with $p< 0.05$ were considered.

3.2.4 siRNA transfections

siRNA transfections were performed using Lipofectamine RNAiMAX Reagent (Invitrogen) following the manufacturer's protocol. Briefly, 5 x 10^5 cells were seeded into six-well tissue culture plates one day prior to transfection with 50 nM IGF1β receptor siRNA (siIGF1Rβ) or a non-targeting control siRNA (siCtl) (Dharmacon, Inc., Lafayette CO). After 6 h, the culture medium in each well was replaced with 2 ml culture medium containing 0.1% FBS, and the cells were further incubated overnight (18 h). The culture medium was then removed and the cells were treated with IGF1 in culture medium containing 0.1% serum for the indicated time periods.

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

Total RNA was prepared using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Single-stranded cDNA was synthesized from 2 µg total RNA according to the manufacturer's procedure (Amersham Biosciences, Quebec, Canada). The primers used for SYBR Green RT-qPCR were as follows: for human E-cadherin, sense, 5'-ACA

GCC CCG CCT TAT GAT T-3' and antisense, 5'-TCG GAA CCG CTT CCT TCA-3'; for Snail, sense, 5'-CCC CAA TCG GAA GCC TAA CT -3' and antisense, 5'-GCT GGA AGG TAA ACT CTG GAT TAG A -3'; for Slug, sense, 5'-TTC GGA CCCACA CAT TAC CT-3' and antisense, 5'-GCA GTG AGG GCA AGA AAA AG-3'; and for GAPDH, sense, 5'-ATG GAA ATC CCA TCA CCA TCT T-3' and antisense, 5'-CGC CCC ACT TGA TTT TGG -3'. RT-qPCR was performed using the Applied Biosystems 7300 Real-Time PCR System equipped with a 96-well optical reaction plate. Relative quantification of mRNA levels was performed using the comparative Cq method (ΔΔCq method) with GAPDH as the reference gene.

3.2.6 Western blot analysis

Cells were harvested in lysis buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 1 mM EDTA, 0.1% SDS] containing protease inhibitor cocktail (Sigma-Aldrich), and protein concentrations were determined using the DC Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA). Protein (40 µg) was electrophoresed on 7.5 % SDS-polyacrylamide gels, transferred to nitrocellulose membranes (Amersham Bioscience), and incubated with specific primary antibodies at 4 ^{o}C overnight. After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h and were visualized with enhanced chemiluminescent substrate (Thermo Fisher Scientific Inc, Waltham, MA).

3.2.7 MTT assay

The cells (5 x 10^4) were seeded in each well in 96-well plate. After 24 h, various concentrations of IGF1 were added for further 48 h. After the incubation, 50 ul of MTT (5mg/ml)

was added into the medium, and the plates were incubated for an additional 2 h. The medium was discarded, and the formazan blue, which was formed in the cells, was dissolved in 50ul DMSO. The optical density was measured at 490 nm using a microplate spectrophotometer (Dynex technologies, Sullyfield, VA, USA).

3.2.8 Data analysis

All experiments were performed at least three times. All values are expressed as mean ± SEM. Data were analyzed by Student's *t*-test or one-way ANOVA followed by Dunnett's *post hoc* test using GraphPad Prism 5 (GraphPad Software, San Diego, CA). $P < 0.05$ was considered statistically significant.

3.3 Results

3.3.1 IGF1 is overexpressed in ovarian cancer

Several lines of evidences have indicated that IGF1 is involved in tumor progression. To determine the clinical relevance of IGF1 expression in human ovarian carcinoma, we used the Oncomine cancer microarray database (Rhodes et al., 2004), which provides publicly available data sets of cancer gene expression for data mining. In Bonome Ovarian data set, IGF1 mRNA levels were higher in the cases then normal ovarian surface epithelium (OSE) (Figure 3.1A) (Bonome et al., 2008). Moreover, in two independent data sets (Figure 3.1B, Anglesio and Tothill Ovarian), a statistically significant increase was present in IGF1 mRNA expression levels in ovarian carcinomas compared to borderline OSE-stromal tumors (Anglesio et al., 2008;Tothill et al., 2008). Furthermore, high level expression of IGF1 was associated with late tumor stage (stage III & IV) (Figure 3.1C) and distant metastasis status (Figure 3.1D). Collectively, our

results suggest that IGF1 acts as an autocrine or paracrine local factor to promote ovarian cancer progression, and a potential diagnostic marker in ovarian cancer.

3.3.2 Effect of IGF1 on E-cadherin expression in ovarian cancer cells

As a first step toward analyzing the role of IGF1 in ovarian cancer progression, we investigated the effect of IGF1 on E-cadherin expression in OVCAR-5 and SKOV-3 cells. Our results showed that treatment with IGF1 down-regulated E-cadherin mRNA levels in both a time- (Figure 3.2A) and dose-dependent manner (Figure 3.2B). Similarly, Western blot analysis showed that treatment with IGF1 down-regulated E-cadherin protein levels in a dose-dependent manner in ovarian cancer cells (Figure 3.2C).

3.3.3 IGF1 down-regulates E-cadherin expression via IGF1 receptor

To further investigate the molecular mechanism by which IGF1 down-regulates E-cadherin expression, we first examined the phosphorylation status of IGF1β receptor (IGF1Rβ) and its downstream signaling pathway Akt/mTOR upon treatment with IGF1 at 5, 15, 30, 60, and 120 minutes post-treatment. We found that IGF1 treatment induced the phosphorylation of IGF1Rβ, Akt (Ser473) ,and activation of mTOR signaling in a time-dependent manner in OVCAR-5 cells, as indicated by phosphorylation of the mTOR downstream molecule, p70S6K (Figure 3.3A). To examine whether the IGF1-induced E-cadherin down-regulation is an IGF1Rβ-dependent action, we used the IGF1Rβ siRNA (siIGF1Rβ) to specifically knockdown IGF1Rβ and block the IGF1-induced Akt/mTOR activation in OVCAR-5 cells (Figure 3.3B). As shown in Figure 3.3C, knockdown of IGF1Rβ markedly diminished the IGF1-induced E-cadherin down-regulation.

3.3.4 IGF1-induced E-cadherin down-regulation via the PI3K/Akt/mTOR pathway

Next, to determine whether the PI3K/Akt/mTOR signaling pathway was involved in the regulation of E-cadherin levels by IGF1, we used the PI3K-specific inhibitor (wortmannin) and the mTOR-specific inhibitor (rapamycin) to specifically block the PI3K/Akt and the mTOR pathways, respectively (Figure 3.4A). As shown in Figure 3.4B, the PI3K and mTOR inhibitors blocked IGF1-induced E-cadherin expression, which demonstrated the involvement of the PI3K/Akt/mTOR pathways in the IGF1-mediated down-regulation of E-cadherin expression in ovarian cancer cells.

3.3.5 Effect of IGF1 on Snail and Slug expression in ovarian cancer cells

To determine if IGF1 down-regulates E-cadherin expression by regulating the transcriptional regulation of E-cadherin, the mRNA levels of the E-cadherin transcriptional repressors, Snail and Slug were examined by RT-qPCR. Treatment with IGF1 significantly increased Snail and Slug mRNA levels in a time- (Figure 3.5A & 3.5B) and dose-dependent manner (Figure 3.5C & 3.5D).

3.3.6 IGF1 up-regulates Snail and Slug expression via the IGF1R/PI3K/Akt/mTOR pathways

To determine whether IGF1Rβ is involved in IGF1-induced increases in Snail and Slug mRNA, OVCAR-5 and SKOV-3 cells were transiently transfected with siIGF1Rβ for 48 h and then treated with IGF1 for a further 3 h. As shown in Figure 3.6A & 3.6B, siIGF1Rβ markedly abolished the IGF1-induced Snail and Slug expression. I further examined the role of the PI3K/Akt/mTOR pathway in IGF1-induced Snail and Slug expression. Treatment with the PI3K

inhibitor (wortmannin) or the mTOR inhibitor (rapamycin) blocked IGF1-induced Snail and Slug up-regulation (Figure 3.6C & 3.6D).

3.3.7 Activation of the IGF1R/PI3K/Akt/mTOR signaling pathway is critical for IGF1-induced cell proliferation

Several lines of evidence indicate that IGF1 plays an important role in the cell proliferation. Thus, we examined the effect of IGF1 on cell proliferation in ovarian cancer cells. OVCAR-5 and SKOV-3 cells were treated with increasing concentrations of IGF1, resulted in a dose-dependent stimulation of proliferation (Figure 3.7A). The involvement of the IGF1R/PI3K/Akt/mTOR signaling pathway on IGF1-stimulated cell proliferation was also evaluated. Our results showed that knockdown of IGF1Rβ reduced the basal cell proliferation and markedly abolished the IGF1-induced cell proliferation (Figure 3.7B). Also, IGF1-induced cell proliferation was blocked by treatment with a PI3K-specific inhibitor (wortmannin) and mTOR-specific inhibitor (rapamycin) (Figure 3.7C). Taken together, these results indicate that the IGF1R/PI3K/Akt/mTOR pathways are involved in IGF1-induced ovarian cancer cell proliferation.

3.4 Discussion

IGF1 is a potent regulator of cell proliferation and survival. The elevated levels of IGF1 and its receptors present in ovarian cancer suggest that IGF1 plays an important role in tumor progression (Brokaw et al., 2007;Conover et al., 1998;Lukanova et al., 2002;Mitsiades and Mitsiades, 2005;Spentzos et al., 2007). However, the role of IGF1 in this process remains to be elucidated. In this study, treatment of IGF1 induced the down-regulation of E-cadherin

expression. Also, our studies suggest that IGF1 exerted its effects in human ovarian cancer cells via the activation of the PI3K/Akt/mTOR signaling pathway and the subsequent induction of transcriptional repressors, Snail and Slug. In addition, we have demonstrated that the PI3K/Akt/mTOR signaling pathway mediated IGF1-induced cell proliferation in ovarian cancer.

Previous studies have demonstrated the presence of the IGF1 pathway in ovarian carcinomas. Ovarian cancer cell lines express all major components of the IGF1 pathway, and exogenous IGF1 administration induces cell proliferation (Conover et al., 1998;Mitsiades and Mitsiades, 2005;Yee et al., 1991). Clinical studies have shown that the higher IGF1 levels in ovarian carcinoma tissues are associated with an increased risk of disease and low overall survival rate (Spentzos et al., 2007). In the current study, data from Oncomine database revealed that higher mRNA levels of IGF1 present in ovarian carcinoma when compared to OSE or borderline OSE-stromal tumor, and the levels of IGF1 are associated with tumor stages and metastasis status, suggesting that endogenous IGF1 may contribute to ovarian tumor progression. This notion is supported by our data that exogenous administration of IGF1 stimulates the ovarian cancer cell proliferation. Furthermore, silencing of IGF1Rβ by siRNA suppressed the basal proliferation and markedly blocked the IGF1-induced cell proliferation *in vitro*. These results are consistent with a previous study demonstrating the IGF1Rβ siRNA inhibited tumor growth and cell proliferation, as well as induced tumor cell apoptosis and suppressed angiogenesis in an OVCAR-3 xenograft model (An et al., 2010).

The PI3K/Akt signaling pathway is well-documented to be frequently deregulated and serves as an oncogenic pathway in ovarian carcinomas (McCluggage, 2011). IGF1 has been shown to activate the PI3K/Akt pathway in various cancer cells (Burroughs et al., 2003;Suzuki and Takahashi, 2000). The current data support a crucial role for the IGF1R/PI3K/Akt/mTOR

signaling pathway in mediating the regulation of E-cadherin by IGF1 in ovarian carcinoma. Aberrant inhibition of IGF1Rβ by siRNA and its downstream PI3K/Akt signaling pathway blocked IGF1-induced E-cadherin down-regulation and cell proliferation. These results are consistent with a previous study that has demonstrated a requirement for PI3K/Akt signaling in E-cadheirn down-regulation in mouse kidney collecting duct epithelial cells (Ivanova et al., 2008). Moreover, the inhibition of the mTOR pathway, which is downstream of PI3K/Akt signaling, also blocked IGF1-induced E-cadherin down-regulation as well as cell proliferation. These results are consistent with a previous study demonstrating a requirement for mTOR signaling in E-cadherin down-regulation in prostate cancer cells (van der Poel, 2004). It has been well documented that the mTOR signaling pathway regulates protein synthesis in response to various growth factors and consequently affects both cell survival and cell proliferation (Martin and Blenis, 2002). In prostate cancer cells, IGF1 has been shown to down-regulate E-cadherin expression and enhance cell invasion via the MAPK/ERK signaling pathway (Graham et al., 2008). Emerging evidence suggests that both MAPK/ERK and PI3K/Akt pathways are involved in the regulation of E-cadherin (Graham et al., 2008;Saegusa et al., 2009), the elucidation of whether the MAPK/ERK pathway influences E-cadherin levels in response to IGF1 in ovarian cancer cells will be of interest. Taken together, our result indicate that IGF1-dependent PI3K/Akt/mTOR activation is involved in E-cadherin down-regulation and ovarian cancer cell proliferation.

In cancer cells, loss of E-cadherin gene expression is mainly due to an overexpression of transcriptional repressors including Snail, Slug and ZEB1 (Bolos et al., 2003;Grooteclaes and Frisch, 2000;Peinado et al., 2007). Indeed, elevated Snail and Slug protein levels have been found in ovarian carcinoma (Elloul et al., 2006). Also, a previous study has shown that the

expression of Snail and Slug were increased stepwise in ovarian benign, borderline and malignant tumors (Yoshida et al., 2009), indicating that high expression of Snail/Slug family members may be involved in advanced stage ovarian carcinoma metastatic progression (Elloul et al., 2005;Imai et al., 2003). A previous study demonstrated that the overexpression of Snail and Slug in SKOV-3 cells resulted in the down-regulation of E-cadherin, enhanced motility and invasiveness (Kurrey et al., 2005). Moreover, Snail and Slug mediated ovarian cancer cell radioresistance and chemoresistance (Kurrey et al., 2009). Interesting, hyperactivation of IGF1R/PI3K/Akt signaling resulted in cisplatin resistance in ovarian cancer cells (Eckstein et al., 2009). In the present study, the inhibition of PI3K/Akt/mTOR signaling, by wortmannin and rapamycin, abolished IGF1-induced Snail and Slug levels expression suggesting that this pathway is critical for their expression. Further studies are required to elucidate whether Snail and Slug mediates IGF1-induced drug resistance in ovarian cancer cells. Snail and Slug expression can be regulated by PI3K/Akt signaling (Ivanova et al., 2008;Saegusa et al., 2009); however, mine is the first study to show that IGF1 can induce Slug mRNA. The activation of PI3K/Akt signaling has been demonstrated to stimulate Snail and Slug expression via GSK-3β/β-catenin signaling and to subsequently down-regulate E-cadherin in different cellular contexts (Ivanova et al., 2008;Saegusa et al., 2009;Wu et al., 2011). It is well known that PI3K/Akt signaling induces nuclear β-catenin accumulation through the inhibition of GSK3β (Cross et al., 1995;Li and Sun, 1998). In addition, it has been demonstrated that the inhibition of mTOR signaling by rapamycin blocks increased β-catenin translocation into the nucleus in human pancreatic β-cells (Liu et al., 2009). Therefore, IGF1 may activate PI3K/Akt signaling and its downstream mediators, such as GSK3β and mTOR, to induce β-catenin-dependent transcription including that of Snail and Slug. Moreover, p70S6K, which is a downstream mediator of mTOR

signaling, has been shown to induce E-cadherin down-regulation via Snail in ovarian cancer cells (Pon et al., 2008). Further studies are required to elucidate the precise mechanism of the elevation of Snail and Slug levels by the mTOR pathway.

In conclusion, our results demonstrated that IGF1 down-regulates E-cadherin expression, most likely through the transcriptional suppression on Snail and Slug, which are concomitantly expressed by the activation of the PI3K/Akt/mTOR. Also, inhibition of either PI3K/Akt/mTOR result in markedly diminished IGF1-induced cell proliferation. Given that IGF1 expression is associated with tumor stage in ovarian cancer patients, the design of therapeutics that target IGF1 signaling may have relevant implications in the treatment of this malignancy.

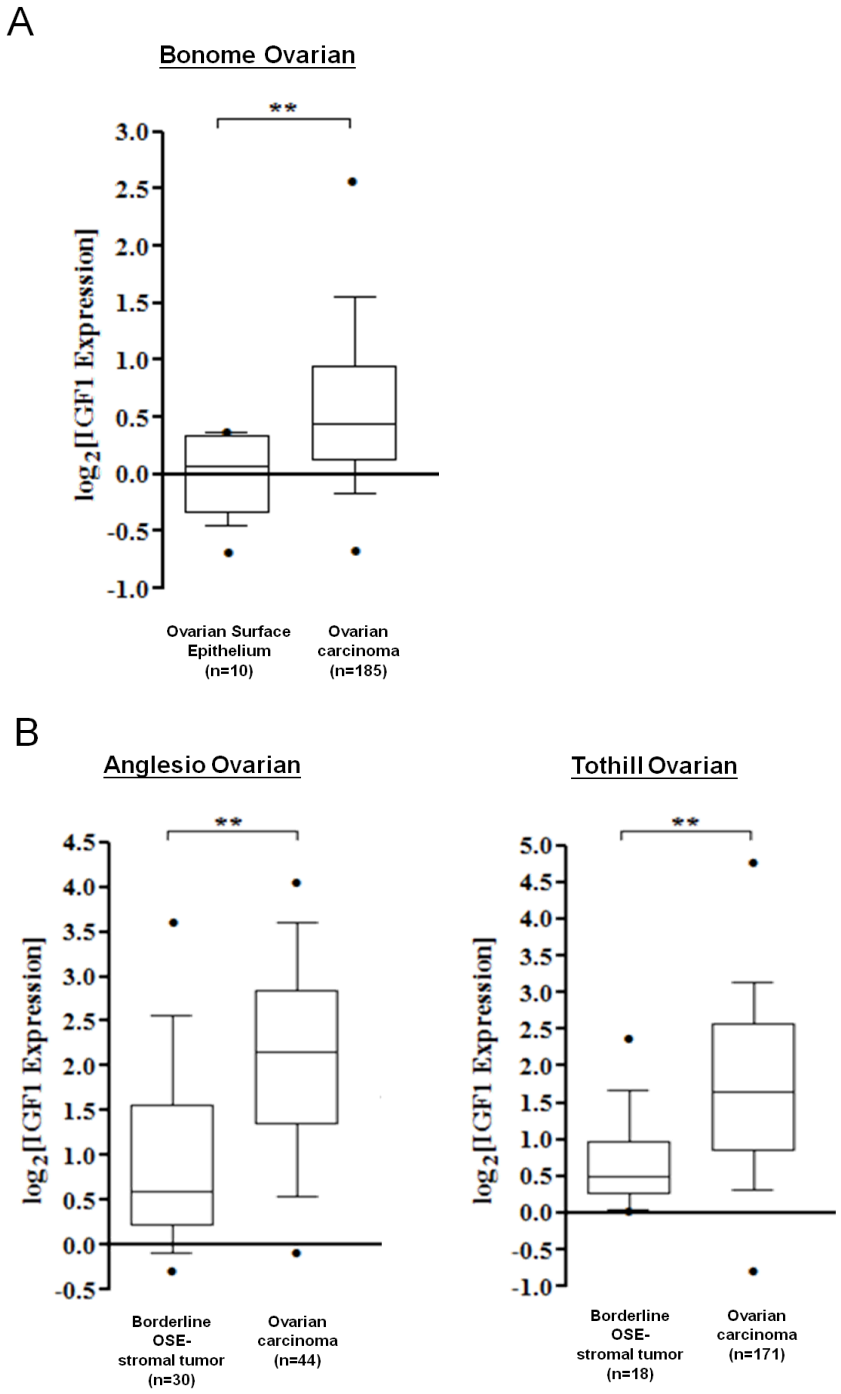


Figure 3.1. IGF1 is overexpressed in human ovarian cancer. (A & B) IGF1 levels are significantly elevated in ovarian carcinoma compared to normal OSE (A) and borderline OSE-stromal tumors (B). Box plot of IGF1 expression in OSE, borderline OSE-stromal tumor and ovarian carcinoma. Dots above and below represent the maximum and minimum expression, respectively. Bars above and below represent the 90th and 10th percentile of expression, respectively. The box delineates the first to third quartiles of expression, and the central bar represents the median. The data obtained from www.oncomine.org and analyzed by Student's *t*-test.

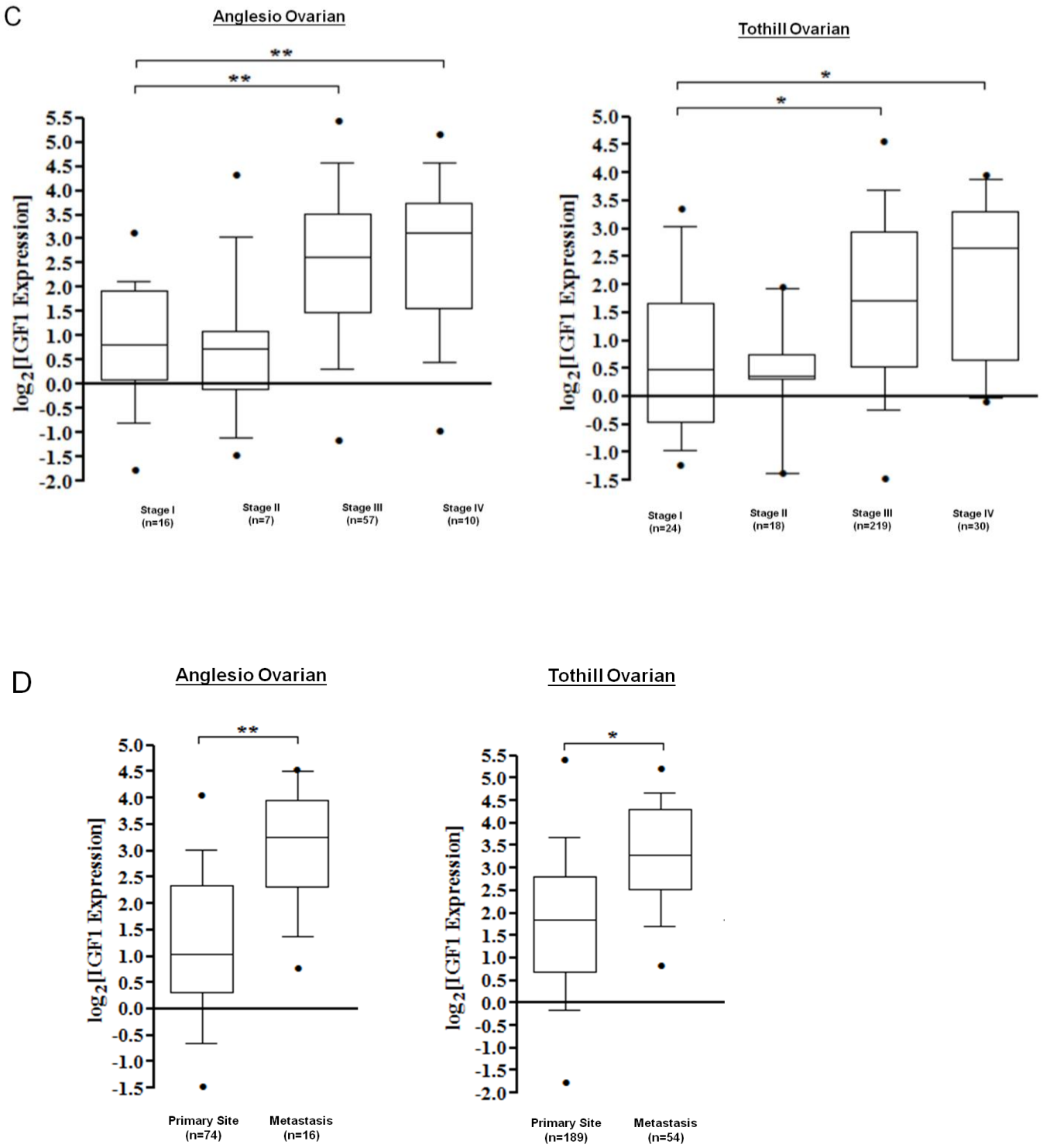


Figure 3.1. (C) In tumor stages, stage I encompasses stages IA, IB and IC; stage II encompasses stages IIA, IIB and IIC; stage III encompasses stages IIIA, IIIB and IIIC. The significance of the results was evaluated by one-way ANOVA followed by Dunnett's *post hoc* test. (D) IGF1 levels are significantly elevated in metastasis samples compared to primary site samples in ovarian carcinoma. The data analyzed by Student's *t*-test (*, $P<0.05$; **, $P < 0.001$).

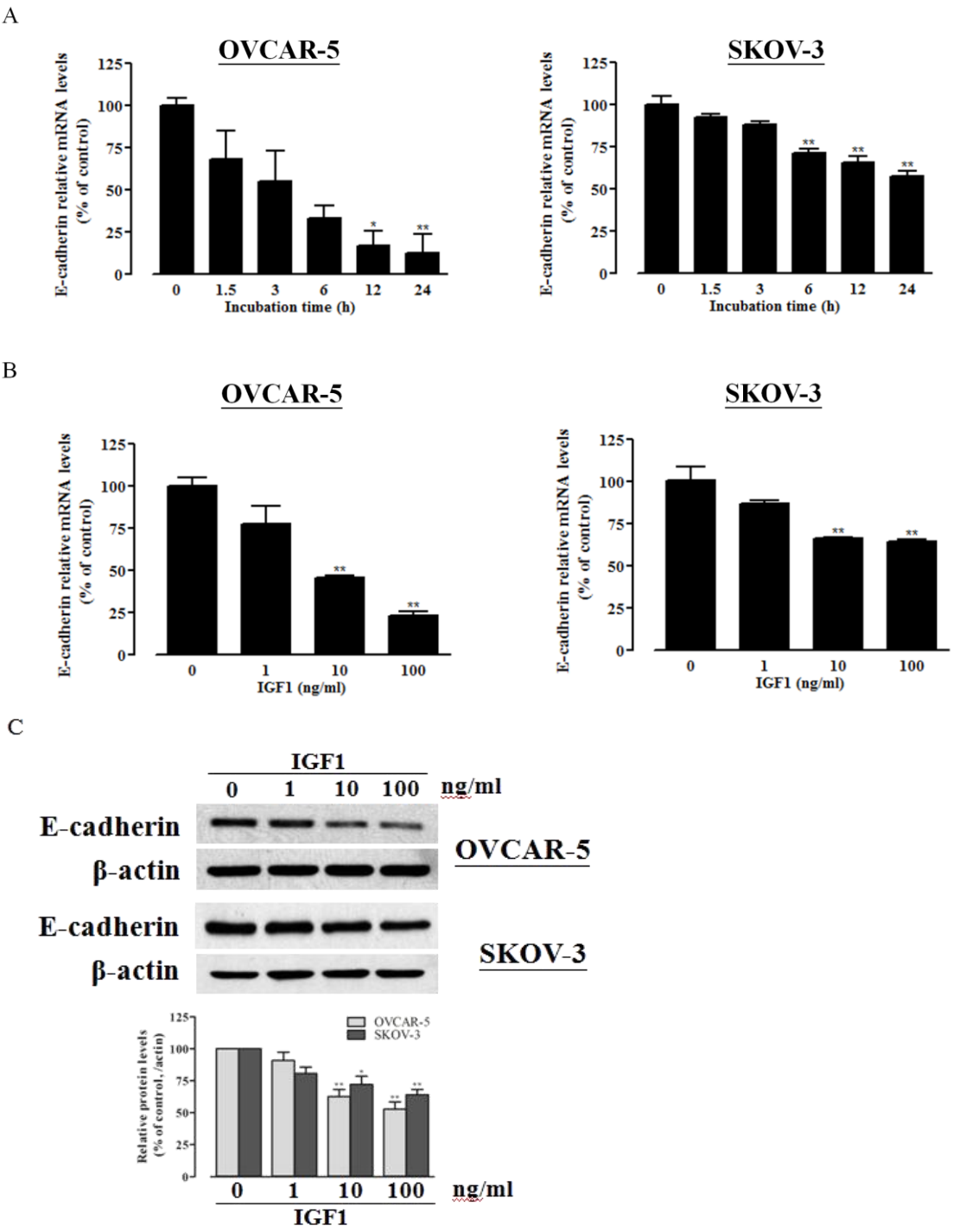


Figure 3.2. IGF1 suppresses E-cadherin mRNA and protein levels in OVCAR-5 and SKOV-3 cells. (A) OVCAR-5 and SKOV-3 cells were treated with 100 ng/ml IGF1 for 0 to 24 h as indicated, and then E-cadherin mRNA levels were analyzed by RT-qPCR. (B and C) OVCAR-5 and SKOV-3 cells were treated with different dose of IGF1 for 24 h after which E-cadherin mRNA levels (B) and protein levels (C) were analyzed by RT-qPCR and Western blotting, respectively. Results represent the mean ± SEM (n=3; *, P<0.05; **, P < 0.001). Data were analyzed by one-way ANOVA followed by Dunnett's *post hoc* test.

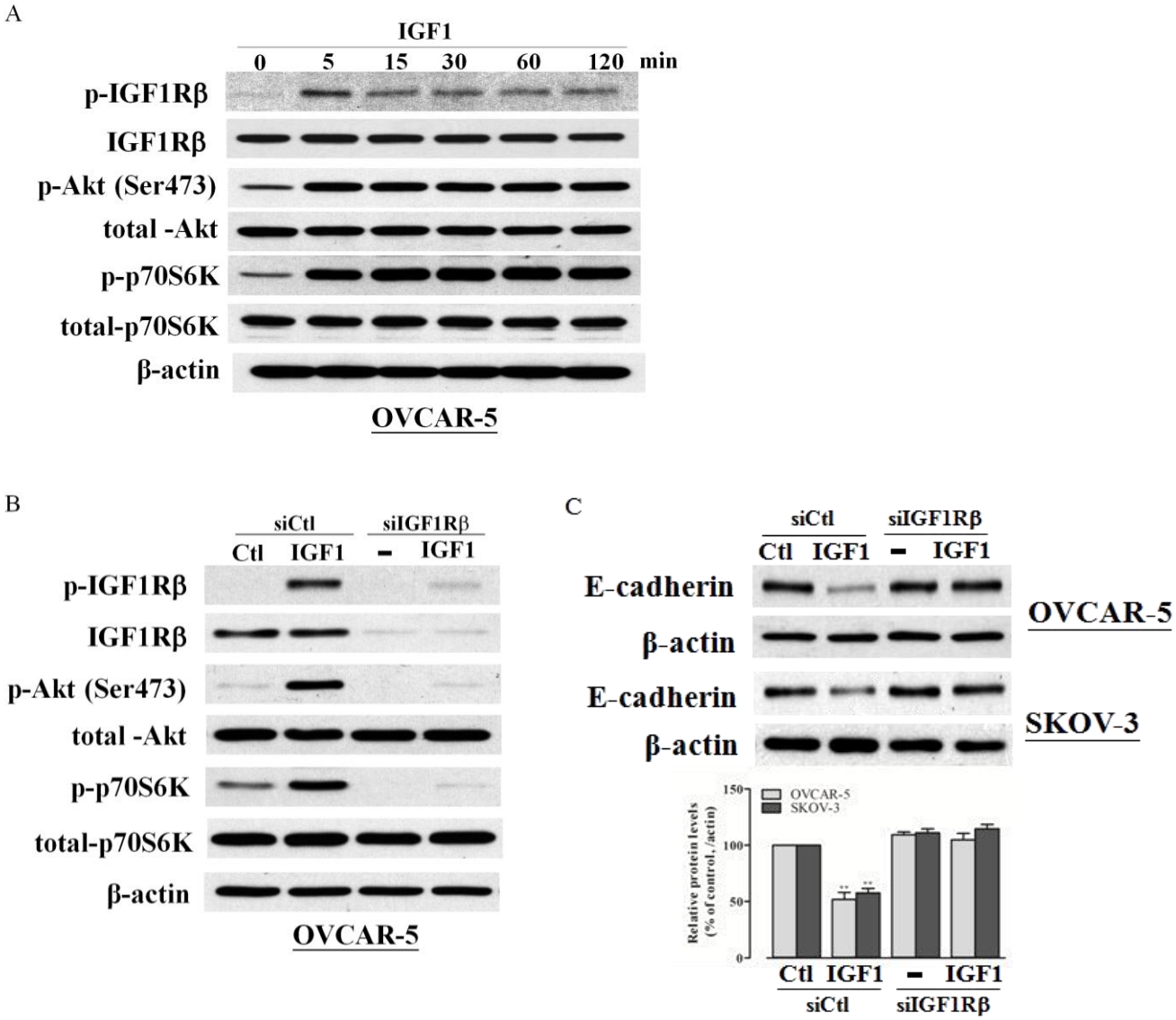


Figure 3.3. IGF1 suppresses E-cadherin via the IGF1 receptor. (A) OVCAR-5 cells were treated with 100 ng/ml IGF for 0 to 120 min as indicated. Phosphorylated and total IGF1 receptor (IGF1Rβ), phosphorylated and total Akt, phosphorylated and total p70S6K, and β-actin levels were analyzed by Western blot analysis. (B) OVCAR-5 cells were transiently transfected with scrambled siRNA (siCtl) or IGF1Rβ siRNA (siIGF1Rβ) for 48 h. After transfection, the cells were treated with 100 ng/ml IGF1 for 30 min. Phosphorylated and total IGF1 receptor (IGF1Rβ), phosphorylated and total Akt, phosphorylated and total p70S6K, and β-actin levels were analyzed by Western blot analysis. (C) OVCAR-5 and SKOV-3 cells were transiently transfected with scrambled siRNA (siCtl) or IGF1Rβ siRNA (siIGF1Rβ) for 48 h. After transfection, the cells were treated with 100 ng/ml IGF1 for 24 h. E-cadherin, and β-actin protein levels were analyzed by Western blotting.

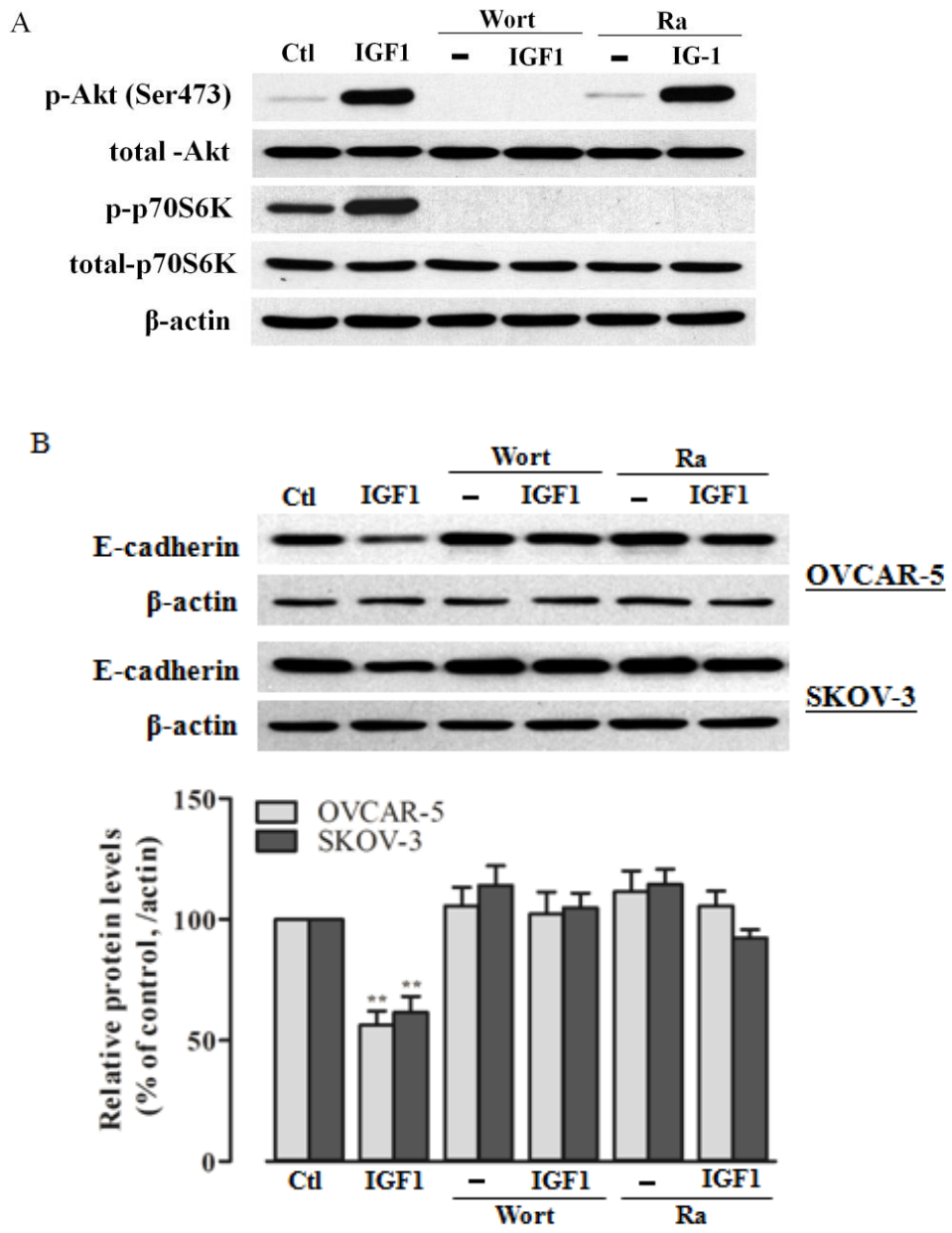


Figure 3.4. IGF1 suppresses E-cadherin expression via the PI3K/Akt/mTOR signaling pathway. (A) OVCAR-5 cells were pretreated with wortmannin (1 μM) or rapamycin (20 nM) for 30 min prior to the addition of 100 ng/ml IGF1 for 30 min. Phospho-Akt and Akt, along with phospho-p70S6K and p70S6K protein levels were analyzed by Western blotting. The β-actin antibody was used as a control for equal loading. (B and C) OVCAR-5 and SKOV-3 cells were pretreated with wortmannin (B) or rapamycin (C) for 30 min prior to addition of 100 ng/ml IGF1 for 24 h, after which E-cadherin protein levels were analyzed by Western blotting.

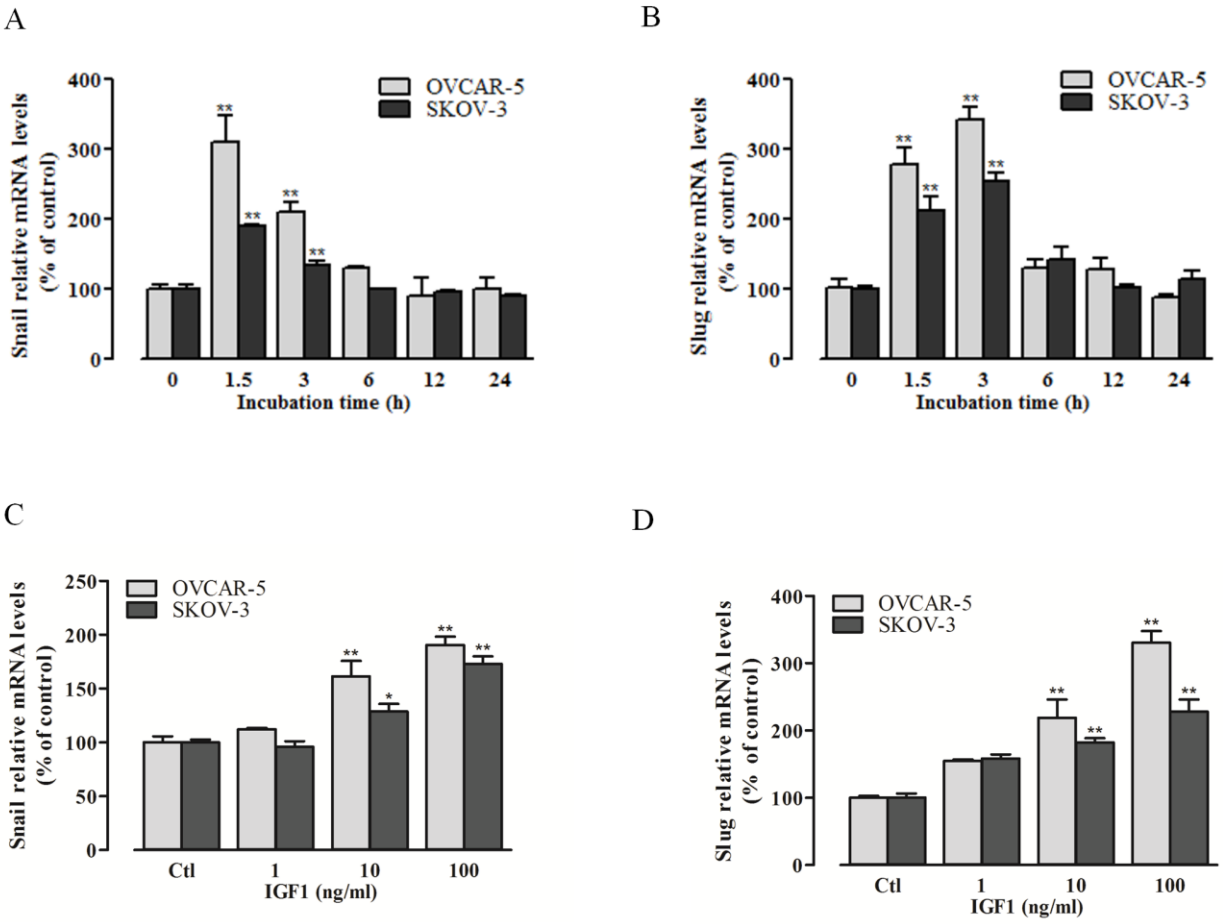


Figure 3.5. IGF1 increases Snail and Slug transcriptional levels in OVCAR-5 and SKOV-3 cells. (A & B) OVCAR-5 and SKOV-3 cells were treated with 100 ng/ml IGF1 for various times, and the mRNA levels of Snail (A) and Slug (B) were analyzed by RT-qPCR. (C & D) OVCAR-5 and SKOV-3 cells were treated with different doses of IGF1 for 3 h, and the Snail (C) and Slug (D) mRNA levels were analyzed by RT-qPCR. Results represent the mean ± SEM (n=3; *, $P<0.05$; **, $P < 0.001$). Data were analyzed by one-way ANOVA followed by Dunnett's *post hoc* test.

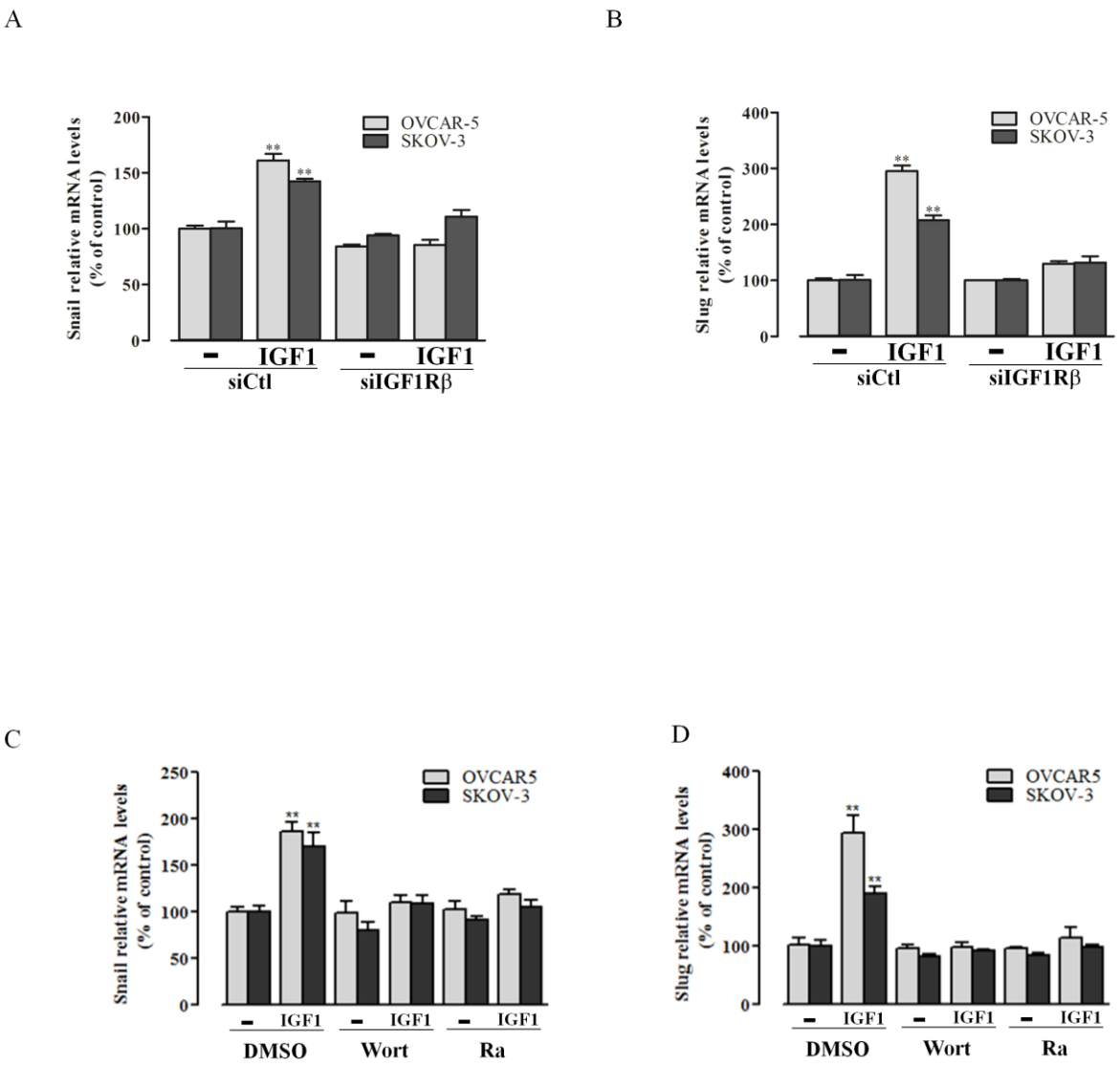


Figure 3.6. IGF1 up-regulates Snail and Slug expression via the IGF1R/PI3K/Akt/mTOR pathways (A & B) OVCAR-5 and SKOV-3 cells were transiently transfected with scrambled siRNA (siCtl) or IGF1Rβ siRNA (siIGF1Rβ) for 48 h. After transfection, the cells were treated with 100 ng/ml IGF1 for 3 h. Snail (A) and Slug (B) mRNA levels were analyzed by RT-qPCR. (C & D) OVCAR-5 and SKOV-3 cells were pretreated with wortmannin (1 μM) or rapamycin (20 nM) for 30 min prior to the addition of 100 ng/ml IGF1 for 6 h. Snail (C) and Slug (D) mRNA levels were analyzed by RT-qPCR. Results represent the mean ± SEM (n=3; **, $P < 0.001$). Data were analyzed by one-way ANOVA followed by Dunnett's *post hoc* test.

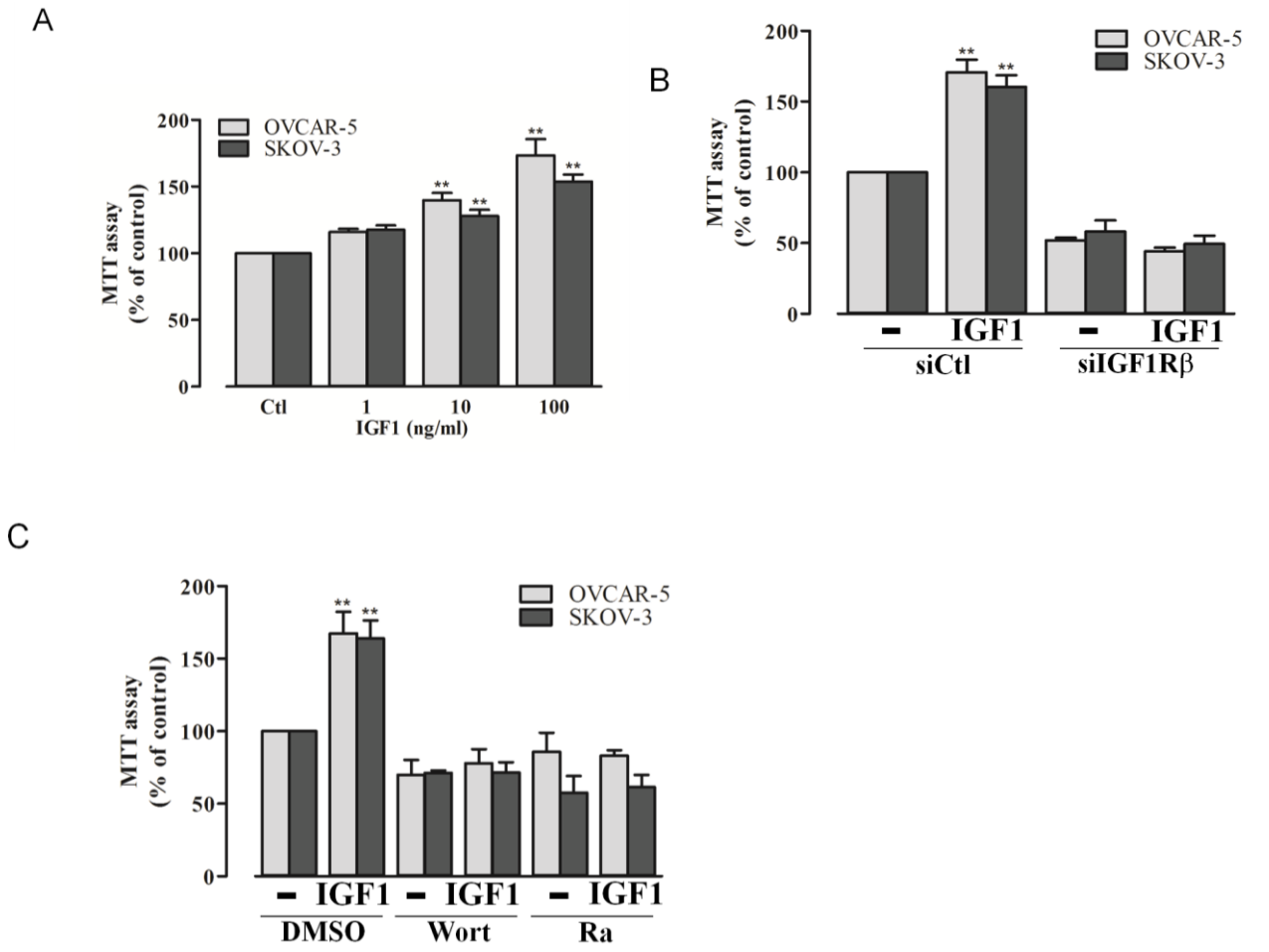


Figure 3.7. Activation of the IGF1R/PI3K/Akt/mTOR signaling pathway is critical for IGF1-induced cell proliferation. (A) OVCAR-5 and SKOV-3 cells were seeded in 96-well plate for 24 h and then treated with different dose of IGF1 for 48 h. (B) OVCAR-5 and SKOV-3 cells were transiently transfected with scrambled siRNA (siCtl) or IGF1Rβ siRNA (siIGF1Rβ) for 48 h. After transfection, the cells were seeded in 96-well plate for 24 h and then treated with 100 ng/ml IGF1 for 48 h. (C) OVCAR-5 and SKOV-3 cells were seeded in 96-well plate for 24 h. The cells were pre-treated with wortmannin (1 μM), rapamycin (20 nM) or U0126 (10 μM) for 30 min prior to the addition of 100 ng/ml IGF1 for 48 h. The cell proliferation was analyzed by MTT assay.Results represent the mean ± SEM (n=3; **, $P < 0.001$). Data were analyzed by one-way ANOVA followed by Dunnett's *post hoc* test.

4. E-cadherin inhibits tumor cell growth by suppressing PI3K/Akt signaling via β-catenin-Egr1-mediated PTEN expression

4.1 Introduction

Generally, cadherins have been studied for their vital roles in cell adhesion. They comprise a superfamily of transmembrane proteins that link adjacent cells via calcium-dependent homophilic interactions (Yagi and Takeichi, 2000). E-cadherin is a tumor suppressor protein whose loss is thought to promote tumor growth and invasion via a mechanism involving β-catenin (Wong and Gumbiner, 2003). β-catenin was originally identified as a cytoplasmic component of adherens junctions, where it associates with E-cadherin and, via α-catenin, the actin cytoskeleton (Geiger et al., 1995;Kemler, 1993). In addition, β-catenin is the main effector of Wnt signaling in the nucleus, where it interacts with lymphoid enhancer factor /T cell factor (LEF/TCF) transcription factors to regulate the expression of genes involved in cell growth control, such as cyclin D1 (Conacci-Sorrell et al., 2003;van Noort and Clevers, 2002). In the absence of Wnt signaling, cytosolic β-catenin is constantly phosphorylated by a degradation complex consisting of glycogen synthase kinase-3β (GSK3β), axin, adenomatous polyposis coli (APC) and casein kinase 1, thereby targeting β-catenin for proteasomal degradation (van Noort and Clevers, 2002). Upon activation of Wnt signaling, GSK3β is inhibited, which leads to the stabilization and nuclear translocation of β-catenin, and the initiation of target gene transcription. E-cadherin-containing adherens junctions ensure that the cytoplasmic pool of β-catenin is maintained at a low level. Thus, E-cadherin could antagonize β-catenin signaling and induce growth inhibition (Gottardi et al., 2001;Shtutman et al., 1999). Indeed, β-catenin has been demonstrated to localize to the nucleus, following the loss of E-cadherin expression (Gottardi et

al., 2001;Onder et al., 2008). However, the detailed mechanism by which the loss of E-cadherin contributes to enhanced β-catenin signaling is not well understood.

Aberrant signaling of the PI3K/Akt pathway has been implicated in the pathogenesis of several human cancers, including epithelial ovarian cancer (Brugge et al., 2007;Woenckhaus et al., 2007). Phosphatase and tensin homolog (PTEN) acts as a tumor suppressor by dephosphorylating phosphotidylinositol-(3,4,5)-triphosphate (PIP3) produced by phosphoinositide-3-kinase (PI3K) (Myers et al., 1998). In human cancers, PTEN is one of the most common targets of mutation or downregulation resulting in the activation of the PI3K/Akt pathway (Blanco-Aparicio et al., 2007). Approximately 27 % of human ovarian cancers display reduced PTEN protein levels and loss-of-function mutations are found in 3-8 % (Bast, Jr. et al., 2009). Overexpression of PTEN suppresses the growth of tumor cells by up-regulating $p27^{Kip1}$ (Furnari et al., 1998;Weng et al., 1999) and downregulating cyclin D1, in an Akt-dependent manner (Radu et al., 2003;Weng et al., 2001). PTEN also modulates migration and proliferation via interaction with cell adhesion molecules such as E-cadherin and β-catenin (Hu et al., 2007;Subauste et al., 2005). It has been shown that PTEN transcription can be transactivated by early growth response gene 1 (Egr1) which binds directly to a consensus Egr1-binding motif in the PTEN promoter (Virolle et al., 2001). Recent studies suggest that E-cadherin modulates PTEN levels in breast cancer cells (Fournier et al., 2009;Li et al., 2007), however the exact mechanism by which E-cadherin regulates PTEN levels is unclear.

In the present study, we demonstrate that E-cadherin regulates tumor cell growth via the PI3K/Akt and β-catenin signaling pathways in epithelial ovarian cancer cells. In the presence of E-cadherin, β-catenin is localized at adherens junctions, PTEN mRNA and protein are high, and PI3K/Akt signaling is reduced. Loss of E-cadherin results in the nuclear translocation of β-

catenin, enhanced β-catenin signaling and reduced Egr1 expression. Egr1 downregulation reduces PTEN, which enhances PI3K/Akt signaling and increases cell growth. Our results point to an interplay between adherens junction assembly and PTEN transcription mediated by the junctional control of β-catenin signaling, and provide important insights into the role of cadherin complexes in cellular proliferation, anchorage-independent growth, and tumor progression.

4.2 Materials and methods

4.2.1 Materials

E-cadherin and β-catenin antibodies were purchased from BD Biosciences (San Jose, CA). Akt, phospho-Akt (Ser 473), cyclin D1, Egr1, phospho-GSK3β (Ser 9), $p27^{Kip1}$ and PTEN antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA). LY294002 was purchased from Calbiochem (San Diego, CA).

4.2.2 Cell culture and transfections

Human ovarian cancer cell lines (A2780, OVCAR-3 and SKOV-3) were purchased from the American Type Culture Collection (ATCC, Manassas, VA), and their use was approved by the University of British Columbia Clinical Screening Committee for Research and Other Studies Involving Human Subjects. Cells were cultured in Medium 199:MCDB 105 (1:1; Sigma-Aldrich Corp., St. Louis, MO) containing 10 % fetal bovine serum (FBS; Hyclone Laboratories Ltd., Logan, UT), 100 U/ml penicillin G and 100 g/ml streptomycin (Life Technologies, Inc., Rockville, MD) in a humidified atmosphere of 5 % CO_2- 95 % air at 37°C. The cells were passaged with 0.06 % trypsin (1:250)/ 0.01 % EDTA in Mg^{2+} /Ca^{2+} - free HBSS at confluence.

4.2.3 Plasmid Constructs

pLKO.1 expression vectors containing short hairpin sequences targeting human E-cadherin (shEcad, GCAGAAATTATTGGGCTCTTT), β-catenin (shβ-cat, GCTTGGAATGAGACTGCTGAT,) and a pLKO.1 scramble shRNA vector were generously provided by Dr. Robert A. Weinberg (Onder et al., 2008). Stable, pooled populations were then generated under puromycin selection (2 μg/ml, Sigma), and clones were isolated by limiting dilution.

An IRES expression vector (Clonetech, Palo Alto, CA) containing full-length wild-type mouse E-cadherin (mEcad) cDNA was a kind gift from Dr. Antonino Passaniti (Sasaki et al., 2000). Stable, pooled populations were then generated under G418 selection (400 μg/ml, Invitrogen Canada, Burlington, ON, Canada), and clones were isolated by limiting dilution.

A degradation-resistant, constitutively activate mutant form of β-catenin (S33Y) was kindly provided by Dr. Eric Fearon (University of Michigan School of Medicine, Michigan). Dominant negative Akt, the T-cell factor (TCF)-binding site reporter plasmid (TOPFLASH), and a mutated control reporter (FOPFLASH) were purchased from Upstate Biotechnology (Lake Placid, NY). Dominant negative TCF repressor (ΔNTCF4) was generously provided by Dr. Hans Clevers (University Hospital, Netherlands). Constitutively active GSK3β (GSK3β-S9A) was a kind gift from Dr. James Woodgett (Ontario Cancer Institute, Toronto, Ontario, Canada). pcDNA-GFP and pcDNA-PTEN-GFP were generously provided by Dr. Alonzo H. Ross (University of Massachusetts Medical School, Massachusetts). The human PTEN promoter constructs (pGL3-PTEN2526/427, pGL3-PTEN1359/427 and pGL3-PTEN1001/427) ligated to the luciferase reporter gene was a gift from Dr. Vuk Stambolic (Ontario Cancer Institute,

University of Toronto, Ontario, Canada) and has been reported previously (Stambolic et al., 2001). pSV-β-galactosidase plasmid was purchased from Promega (Madison, WI).

The mutant pGL3-PTEN2526/427 (mutEgr1) reporter construct was made by directed mutagenesis using the Quick-Change kit (Stratagene, La Jolla, CA) following the manufacturer's protocol. To create the mutEgr1 construct, the PTEN2526/427-*luc* Egr1 site was changed to an *Eco*R1 restriction site using the following oligonucleotide 5'- AGG CGC CCG GGC TCC CGG CGA ATT CGC GGA GGG GGC GGG CAG GCC GGC GGG CGG TGA TGT -3', and its reverse complement (Virolle et al., 2001).

All transfections were carried out using Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's protocol.

4.2.4 Reverse transcription quantitiative real-time PCR (RT-qPCR)

Total RNA was prepared using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Single-stranded cDNA was synthesized from 2 μg of total RNA according to the manufacturer's procedure (Amersham Biosciences, Quebec, Canada). The primers used for SYBR Green RT-qPCR were as follows: for human PTEN, sense, 5'- CCG TTA CCT GTG TGT GGT GAT ATC -3' and antisense, 5'- GAA TGT ATT TAC CCA AAA GTG AAA CAT T -3'; for GAPDH, sense, 5'-ATG GAA ATC CCA TCA CCA TCT T-3' and antisense, 5'-CGC CCC ACT TGA TTT TGG -3'. RT-qPCR was performed using the Applied Biosystems 7300 Real-Time PCR System equipped with a 96-well optical reaction plate. Relative quantification of mRNA levels was performed using the comparative Cq method (ΔΔCq method) with GAPDH as the reference gene.

4.2.5 Western blot analysis

Cells were harvested in lysis buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 1 mM EDTA, 0.1% SDS] containing protease inhibitor cocktail (Sigma), and protein concentrations were determined using the DC Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA). Protein (40 μg) was electrophoresed on 7.5 % SDS-polyacrylamide gels, transferred to nitrocellulose membranes (Amersham Bioscience), and incubated with specific primary antibodies at 4 ^{o}C overnight. After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h and visualized with enhanced chemiluminescent substrate (Thermo Fisher Scientific Inc, CA).

4.2.6 Soft agar growth assays

2 x 10^4 cells were suspended in M199:MCDB 105 supplemented with 10 % heat-inactivated FBS, penicillin/streptomycin, and 1.2 % low melting point agarose (Invitrogen). Suspensions were plated on 60-mm culture dishes pre-coated with a layer of M199:MCDB 105 supplemented with 10 % heat inactivated FBS, penicillin/streptomycin, and 1.2 % low melting point agarose. After 14 – 21 d in culture, colonies were stained with a 0.005 % crystal violet solution and counted. When indicated, DMSO (control) or PI3K inhibitor (LY294002, 10 μM) was added to the suspended cells and replaced every other day with fresh medium containing the respective compounds.

4.2.7 Cell growth assay

Cells growing under standard conditions were harvested, counted, and resuspended in complete medium at 5×10^3 cells per well in 12-well plates. At the indicated times after plating, cells were trypsinized, mixed 1:1 with Trypan blue and counted using a hemacytometer.

4.2.8 Suspension cell growth assay

Poly-2-hydroxyethyl methacrylate (poly-HEMA; Sigma) was applied to 24-well plates (0.2 ml/well of a 12 mg/ml stock solution in ethanol) and allowed to air dry. SKOV-3/shCtl, shEcad and mEcad cells (5×10^4/well) were cultured on poly-HEMA-coated plates for 24 or 48 h and viable cells were counted with Trypan blue.

4.2.9 Luciferase Assay for TCF Activities

Two different TCF luciferase reporter genes were used in this assay: an intact wild-type TCF-luciferase reporter construct (TOPFLASH), and a mutated TCF-luciferase reporter construct (FOPFLASH), which served as a negative control for TOPFLASH activity (Korinek et al., 1997). Cells were seeded in 6-well plates and transiently transfected with 0.5 μg of the TOPFLASH or FOPFLASH reporter plasmid, along with 0.5 μg of pcDNA 3.1, dominant negative Akt (DN-Akt), or constitutive active GSK3β (GSK3β-S9A). As a control for transfection efficiency, 0.25 μg of a β-galactosidase construct was included in each transfection. Cells were harvested 48 h after transfection and extracts were prepared with 200 μL of reporter lysis buffer (Promega). Luciferase and β-galactosidase activity were assayed according to the manufacturer's protocol using a Luciferase Assay Kit and β-galactosidase Enzyme Assay system,

respectively (Promega). Luciferase activity in each well was normalized to the β-galactosidase activity. To allow easier comparison of the transcriptional activities, the background transcriptional activity represented by the normalized FOPFLASH value was subtracted from the normalized TOPFLASH value. Three independent experiments, each assayed in triplicate, were performed on separate cell passages.

4.2.10 Data analysis

All the experiments were performed at least three times. All values are expressed as mean ± SEM. Data were analyzed by Student's *t*-test or one-way ANOVA followed by Tukey's *post hoc* test using GraphPad Prism 5 (GraphPad Software, San Diego, CA). $P < 0.05$ was considered statistically significant.

4.3 Results

4.3.1 Loss of E-cadherin induces cell growth in human ovarian cancer cells

Initially, we tested whether E-cadherin mediates anchorage-independent growth. A2780, OVCAR-3 and SKOV-3 cells, which express different levels of endogenous E-cadherin protein (Figure 4.1A), were cultured in soft agar to test their capacities for anchorage-independent growth. Our results show that endogenous levels of E-cadherin are inversely correlated with the capacity for anchorage-independent growth in these cell lines (Figure 4.1B). To further confirm the role of E-cadherin in suppressing anchorage-independent growth, OVCAR-3 and SKOV-3 cells were stably transfected with pLKO.1 expression vectors encoding short hairpin sequences targeting human E-cadherin. As shown in Figure 4.2A, stable knockdown of endogenous E-cadherin increased anchorage-independent growth in OVCAR-3 and SKOV-3 cells. In contrast,

the expression of exogenous murine E-cadherin (mEcad) suppressed anchorage-independent growth in A2780 and SKOV-3 cells (Figure 4.2B). Effects observed on anchorage-independent growth corresponded to the extent of E-cadherin downregulation or overexpression in SKOV-3 cells and A2780 cells, respectively (Figure 4.2A and 4.2B).

To confirm these results, stably transfected SKOV-3 cells were cultured in suspension in poly-HEMA-coated dishes to prevent cell substratum attachment. The viability of control cells declined slightly over a 2-day period, whereas mEcad cells exhibited a dramatic decrease in viable cell number and shEcad cells continued to grow (Figure 4.2C). In addition, transient re-expression of murine E-cadherin suppressed the increased growth of stably transfected SKOV3/shEcad cells in suspension culture (data not shown). Next, we examined the effects of E-cadherin modulation on the growth of adherent cells. Stably transfected SKOV-3/shEcad cells exhibited a faster growth rate than control cells, with significant differences observed at 48, 72 and 96 h (Figure 4.2D). In contrast, mEcad cells grew slower than control cells with significant differences observed at 72 and 96 h. Taken together, these results indicate that endogenous E-cadherin suppresses the growth of ovarian cancer cells *in vitro.*

4.3.2 Loss of E-cadherin promotes anchorage-independent growth via PI3K/Akt mediated β-catenin/TCF signaling in human ovarian cancer cells

Previous studies have indicated that E-cadherin-mediated cell-cell adhesion transiently activates the PI3K/Akt signaling pathway in ovarian carcinoma cell lines (De Santis et al., 2009;Reddy et al., 2005). Thus, we next examined whether stable changes in E-cadherin expression modulate PI3K/Akt signaling by determining the phosphorylation level of Akt at Ser473. Our results show that depletion of endogenous E-cadherin increases basal

phosphorylation of Akt in both OVCAR-3 and SKOV-3 cells (Figure 4.3A). PI3K/Akt signaling is known to lead to the phosphorylation and inactivation of glycogen synthase kinase-3β (Cross et al., 1995), and recent data have shown that E-cadherin loss inhibits GSK3β activation by inducing its phosphorylation (Onder et al., 2008). Thus, we hypothesized that E-cadherin-depletion-mediated activation of PI3K/Akt signaling may lead to the phosphorylation and inhibition of GSK3β. Consistent with this hypothesis, we found that E-cadherin depletion increased the levels of phosphorylated GSK3β in both SKOV-3 and OVCAR-3 cells (Figure 4.3A). In addition, E-cadherin-depleted cells exhibited increased levels of cyclin D1 and reduced levels of $p27^{Kip1}$, known targets of Akt signaling involved in cell-cycle control. In contrast, expression of murine E-cadherin suppressed Akt and GSK3β phosphorylation, reduced cyclin D1 and increased $p27^{Kip1}$ protein levels (Figure 4.3A). These data show that the Akt signaling pathway is activated in E-cadherin-depleted human ovarian cancer cells.

The observation that E-cadherin depletion induces Akt signaling and growth in soft agar suggested the possibility that loss of E-cadherin promotes anchorage-independent growth through induction of the PI3K/Akt signaling pathway. To test this hypothesis, we treated SKOV-3 control cells (shCtl) and E-cadherin-depleted cells (shEcad) with the PI3K inhibitor LY294002 (10 μM). Treatment with LY294002 abolished the shEcad-mediated increases in phosphorylated Akt and cyclin D1, but had no effect on the reductions in $p27^{Kip1}$ (Figure 4.3B). Moreover, treatment with LY294002 completely abolished the increase in phosphorylated GSK3β observed in E-cadherin-depleted SKOV-3 cells (Figure 4.3B), thus suggesting a role for PI3K/Akt in GSK3β inactivation following the loss of E-cadherin. Functionally, LY294002 abolished the increase in cell growth in soft agar induced by E-cadherin depletion (Figure 4.3D). These data

strongly indicate that loss of E-cadherin promotes the growth of SKOV-3 cells by activating the PI3K/Akt signaling pathway.

Because E-cadherin has been shown to inhibit β-catenin signaling (Conacci-Sorrell et al., 2003;Gottardi et al., 2001;Stockinger et al., 2001), we therefore examined the effects of E-cadherin loss on β-catenin signaling. Downregulation of E-cadherin in SKOV-3 cells resulted in the loss of β-catenin from sites of cell-cell contact, as assessed by immunocytochemistry (Figure 4.3E). As inactivation of GSK3β has been shown to enhance β-catenin protein stability and transactivation activity (Polakis, 1999), we next investigated whether PI3K/Akt/GSK3β signaling was involved in E-cadherin-depletion-mediated changes in the subcellular localization of β-catenin. A LEF/TCF promoter luciferase reporter system was used to confirm the nuclear translocation and transactivation activity of β-catenin, and to examine the involvement of PI3K/Akt/GSK3β signaling (Figure 4.3F). LEF/TCF promoter activity was increased in SKOV-3/shEcad cells and was abolished by LY294002 treatment. To further demonstrate a vital role for PI3K/Akt-mediated GSK3β inhibition in the activation of β-catenin/TCF-dependent transcription in SKOV-3 cells, we used a dominant negative Akt and a constitutively active form of GSK3β (GSK3β-S9A) in which Ser9 was replaced with alanine, thus preventing phosphorylation and inactivation of the kinase (Eldar-Finkelman et al., 1996;Stambolic and Woodgett, 1994). Forced expression of either dominant negative Akt or GSK3β-S9A abolished the effects of E-cadherin loss on LEF/TCF promoter activity (Figure 4.3F). Taken together, these data strongly implicate the inactivation of GSK3β by the PI3K/Akt pathway in the enhancement of β-catenin/TCF-dependent signaling in response to reduced levels of E-cadherin.

To determine whether enhanced β-catenin signaling mediates increased growth in response to E-cadherin-depletion, we used a short hairpin construct to stably knockdown β-catenin expression in SKOV-3 and SKOV-3/shEcad cells. Downregulation of β-catenin in SKOV-3/shCtl cells resulted in reduced levels of cyclin D1 and increased levels of $p27^{Kip1}$ (Figure 4.3C). Moreover, shEcad-mediated increases in cyclin D1, and reductions in $p27^{Kip1}$, were inhibited by β-catenin knockdown (Figure 4.3C). Importantly, β-catenin knockdown in SKOV-3/shEcad cells completely abolished E-cadherin-depletion-induced anchorage-independent growth (Figure 4.3D). These data suggest that β-catenin signaling is required for the enhanced growth of SKOV-3 cells in response to E-cadherin-depletion.

4.3.3 Loss of E-cadherin inhibits PTEN transcription via Egr1 downregulation

To more precisely define the mechanism by which E-cadherin depletion induces Akt activation, we further examined the signaling upstream of Akt. In particular, we investigated the mRNA and protein levels of PTEN in control and shEcad transfected SKOV-3 and OVCAR-3 cells. As shown in Figure 4.4A, PTEN mRNA levels were downregulated by E-cadherin loss. In addition, PTEN protein levels were also reduced by E-cadherin loss and this effect could be reversed by overexpression of mouse E-cadherin (Figure 4.4B and 4.4C). Similarly, PTEN promoter activities were repressed by E-cadherin loss and could be restored by mouse E-cadherin overexpression in OVCAR-3 and SKOV-3 cells (Figure 4.4D), suggesting that E-cadherin can regulate PTEN at the transcriptional level. Since transcription of PTEN can be transactivated by Egr1, via binding to an Egr1-binding site in the PTEN promoter (Virolle et al., 2001), we examined the protein levels of Egr1 in transfected SKOV-3 and OVCAR-3 cells. Egr1 protein levels were reduced in E-cadherin depleted cells, and could be restored by

overexpression of mouse E-cadherin (Figure 4.4B and 4.4C), suggesting that Egr1 may mediate the effects of E-cadherin on PTEN transcription. To determine whether Egr1 is involved in E-cadherin-mediated PTEN transcriptional regulation, we next examined the effects of E-cadherin downregulation on the activity of PTEN promoter constructs with serial deletions or mutation of the Egr1-binding site. As shown in Figure 4.4E, the luciferase activities of different 5' truncated PTEN promoter constructs that contain the Egr1-binding site were reduced by E-cadherin loss, whereas the luciferase activity of a full-length construct with a mutated Egr1-binding site, pGL3-PTEN2526/427(mutEgr1), was low in both control and shEcad transfected SKOV-3 cells. Since E-cadherin regulates PTEN, which has previously been implicated in the suppression of cell growth (Ramaswamy et al., 1999;Sun et al., 1999), we investigated whether the overexpression of PTEN regulates cyclin D1 and $p27^{Kip1}$ protein levels. Transient transfection of SKOV-3 cells with PTEN decreased cyclin D1 and increased $p27^{Kip1}$ protein levels (Figure 4.4F). Taken together, these results suggest that E-cadherin downregulation reduces PTEN transcription via the downregulation of Egr1, thus leading to reduced PTEN protein levels, enhanced Akt signaling and increased anchorage-independent growth.

4.3.4 Loss of E-cadherin inhibits PTEN transcription via β-catenin/TCF-mediated Egr1 downregulation

The observation that Egr1 and PTEN levels are decreased in SKOV-3/shEcad cells displaying nuclear β-catenin and strong β-catenin-mediated LEF/TCF transactivation led us to investigate whether the low PTEN levels were the result of β-catenin-mediated suppression of Egr1. Lithium chloride (LiCl), which mimics Wnt/β-catenin signaling by inhibiting GSK3β activity and inducing GSK3β phosphorylation, was used to activate β-catenin signaling (van

Noort and Clevers, 2002). Activation of β-catenin signaling, which was confirmed by LEF/TCF promoter luciferase reporter (Figure 4.5A), suppressed PTEN and Egr1 protein levels (Figure 4.5B). Next, we used a constitutively active GSK3β (GSK3β-S9A) construct to confirm the role of GSK3β inactivation in modulating Egr1 and PTEN levels. Expression of GSK3β-S9A induced Egr1 and PTEN protein levels (Figure 4.5C). We also investigated the role of GSK3β inhibition on PTEN promoter activity and found that LiCl suppressed, whereas GSK3β-S9A enhanced, promoter activity SKOV-3 cells (Figure 4.5D). Interestingly, LiCl and GSK3β-S9A did not affect the activity of the Egr-1 mutant PTEN promoter construct (Figure 4.5D), indicating that GSK3β inhibition suppresses transcription of the PTEN gene via Egr1.

Next, we used SKOV-3/shβ-cat and shEcad + shβ-cat clones to assess whether β-catenin regulates Egr1 and PTEN expression. Downregulation of β-catenin resulted in increased Egr1 and PTEN levels in SKOV-3/shβ-cat cells compared with control cells (Figure 4.6A, compare lane 2 to lane 1). Downregulation of β-catenin in SKOV-3/shEcad cells abolished the suppression of Egr1 and PTEN levels induced by E-cadherin-depletion (Figure 4.6A, compare lane 1 to lane 3 vs. lane 2 to lane 4). To further examine the role of β-catenin signaling in the inhibition of Egr1 and PTEN expression, we overexpressed stabilized, constitutively active S33Y β-catenin to activate β-catenin signaling. Activation of β-catenin signaling reduced Egr1 and PTEN protein levels in OVCAR-3 and SKOV-3 cells (Figure 4.6B). In addition, we transfected SKOV-3/shβ-cat cells with S33Y β-catenin and dominant negative TCF. Stabilized S33Y β-catenin was able to reverse the induction of PTEN protein levels in SKOV-3/shβ-cat cells, and this effect could be blocked by dominant negative TCF (Figure 4.6C). Similarly, stabilized S33Y β-catenin reduced PTEN promoter activity, and this effect could be reversed by dominant

negative TCF (Figure 4.6D). We also examined whether Egr1 is involved in β-catenin/TCF-mediated PTEN suppression. Stabilized S33Y β-catenin and dominant negative TCF did not affect the activity of the Egr1 mutant PTEN promoter construct (Figure 4.6D). Collectively, these data suggest that E-cadherin induces PTEN up-regulation and suppresses the PI3K/Akt pathway by β-catenin/TCF-mediated suppression of Egr1, a positive regulator of PTEN transcription.

4.3.5 Regulation of PTEN levels by cell density and E-cadherin-cadherin interactions

Previous studies in colon cancer cell lines have shown that E-cadherin levels increase in dense compared with sparse cultures, and that this depends on the junctional control of β-catenin signaling (Conacci-Sorrell et al., 2003). Therefore, we next examined whether cell density influences E-cadherin levels and whether such changes in E-cadherin contribute, in turn, to the subsequent regulation of PTEN levels in a β-catenin dependent manner. To test this hypothesis, OVCAR-3 and SKOV-3 cells were seeded at different densities (sparse, 6 $x10^3$ cells/cm^2; dense, 6 x 10^4 cells/cm^2) and E-cadherin, Egr1, PTEN and pAkt levels were analyzed by Western blot. Both ovarian cancer cell lines showed increases in E-cadherin, Egr1 and PTEN levels in dense compared with sparse cultures (Figure 4.7A). Dense cultures also displayed a reduced activation of PI3K/Akt signaling (Figure 4.7A). We also tested whether enhanced β-catenin signaling contributes to decreased Egr1 and PTEN levels in sparse cultures. β-catenin depletion in sparse cultures of SKOV-3 cells resulted in increased levels of E-cadherin, Egr1 and PTEN, and reduced levels of PI3K/Akt signaling (Figure 4.7A), suggesting that β-catenin signaling regulates E-cadherin, Egr1 and PTEN levels.

We also examined whether the assembly of adherens junctions in dense SKOV-3 cultures is involved in inducing Egr1 and PTEN expression. To inhibit E-cadherin–dependent adherens junction assembly, dense cultures were seeded in the presence of a monoclonal antibody against the extracellular domain of E-cadherin that is known to block E-cadherin-cadherin interactions. The localization of β-catenin underwent a dramatic change, with β-catenin relocalizing to the nuclei of cells, and with little β-catenin remaining in adherens junctions (Figure 4.7B). Consistent with our shEcad findings, Egr1 and PTEN protein levels were reduced in SKOV-3/shCtl cells incubated with anti-E-cadherin antibody (Figure 4.7C). In contrast, treatment with the anti-E-cadherin antibody did not reduce Egr1 and PTEN protein levels in cells with reduced β-catenin signaling (SKOV-3/shβ-cat; Figure 4.7C). Taken together, these results suggest that E-cadherin, via the assembly of adherens junctions, regulates Egr1 and PTEN expression by modulating β-catenin signaling.

4.4 Discussion

E-cadherin is known to suppress tumor cell growth and invasion, and re-expression of E-cadherin in E-cadherin-deficient carcinomas reverts cells to a less invasive, less aggressive phenotype (Gottardi et al., 2001;Soto et al., 2008;St Croix et al., 1998;Yanagisawa and Anastasiadis, 2006). On the other hand, the expression of E-cadherin supports cohesive, collective cell migration/invasion (Friedl and Gilmour, 2009). While the loss of E-cadherin expression or function is a common event in tumor progression (Nollet et al., 1999;Yap, 1998), there is emerging evidence that the expression of E-cadherin during cancer progression may be dynamic and highly contextual (Kowalski et al., 2003). To date, the role of E-cadherin in ovarian cancer progression remains controversial, particularly in regards to its effects on cell growth. In

the current study, we examined the mechanism by which E-cadherin suppresses tumor cell growth. Our data reveal that endogenous E-cadherin suppresses cell growth via a mechanism involving β-catenin and PI3K/Akt signaling. In particular, we show for the first time that loss of E-cadherin induces β-catenin signaling which represses Egr1-mediated PTEN transcription and leads to the activation of PI3K/Akt signaling in ovarian cancer cells.

Recent studies have suggested that E-cadherin suppresses cell growth by inhibiting β-catenin signaling (Gottardi et al., 2001;Maher et al., 2009;Stockinger et al., 2001), we report here that shRNA-mediated depletion of E-cadherin resulted in relocalization of β-catenin from the membrane to the nucleus and activation of β-catenin-TCF signaling which in turn regulates cell growth in ovarian cancer cells. Theoretically, translocation of β-catenin to the nucleus leads to its association with TCFs and results in regulated transactivation of genes containing the LEF-1/TCF-4 binding sequence near their promoter, such as cyclin D1 (Lin et al., 2000;Morin, 1999;Shtutman et al., 1999). This theory is supported by our finding that the loss of E-cadherin in SKOV-3 cells resulted in increased activation of the TCF promoter–reporter construct, and was associated with increased levels of cyclin D1. In addition to the release of β-catenin from cell adherens junctions, loss of E-cadherin led to the inactivation of GSK3β by phosphorylation. It is well known that the PI3K/Akt signaling can regulate nuclear β-catenin through inhibition of GSK3β (Cross et al., 1995;Li and Sun, 1998). In this study, we show that the loss of E-cadherin induces the activation of PI3K/Akt signaling resulting in the phosphorylation and inactivation of GSK3β, thus reducing the degradation of β-catenin and enhancing β-catenin signaling. Dysregulated PI3K/Akt signaling as well as β-catenin expression and signaling are crucial in tumorigenesis (Chalhoub and Baker, 2009;Morin, 1999).

My findings demonstrate that both β-catenin signaling and PI3K/Akt signaling are required for increased ovarian cancer cell growth in response to the sustained loss of E-cadherin. In contrast, two earlier studies in ovarian cancer cells found that *de novo* formation of homophilic E-cadherin interactions, following calcium deprivation and subsequent calcium restoration, resulted in a transient activation of PI3K/Akt signaling (De Santis et al., 2009;Reddy et al., 2005). These studies also demonstrated that treatment with E-cadherin siRNA or inhibitory antibodies reduced PI3K/Akt signaling and suppressed cell growth; however, the effects of such treatments on β-catenin signaling were not examined. This is important because our data show that β-catenin signaling is required for increased cell growth and reduced PTEN expression in response to the loss of E-cadherin. Interestingly, it has been reported that treatment with E-cadherin siRNA does not alter LEF/TCF reporter gene activity in RMUG-S ovarian cancer cells (Sawada et al., 2008). Moreover, a study found that LEF-1 reporter gene activity and proliferation were not affected when OVCAR-3 cells were engineered to overexpress a dominant-negative E-cadherin chimera which was still able to bind β-catenin (Wu et al., 2008a). Thus, while our results point to an important role for β-catenin signaling in mediating the growth-enhancing effects of E-cadherin loss; it also seems likely that cell growth may not be affected when the loss of E-cadherin function is uncoupled from enhanced β-catenin signaling.

My results indicate that the loss of E-cadherin reduces PTEN levels in ovarian cancer cells, thus leading to increased PI3K/Akt signaling. In agreement, recent studies in breast cancer cells have implicated E-cadherin mediated cell-cell adhesion in the regulation of PTEN. Specifically, exogenous expression of E-cadherin increases, whereas function-blocking E-cadherin antibody or siRNA-mediated knockdown reduces, PTEN protein levels (Fournier et al., 2009;Li et al., 2007). PTEN transcription is regulated by numerous transcription factors.

MEKK4 and JNK promote cell survival by suppressing PTEN transcription via direct binding of NFκB to the PTEN promoter (Xia et al., 2007). In contrast, p53 activates PTEN transcription by directly binding to a p53-binding element in the PTEN promoter (Stambolic et al., 2001). Egr1 has been reported to be a crucial PTEN transactivator by directly binding to the PTEN 5'-untranslated region (Virolle et al., 2001). In the present study, we show that Egr1 provides a critical link between β-catenin/TCF signaling, induced by the loss of E-cadherin, and PTEN transcription. First, mimicking the activation of β-catenin signaling by GSK3β inactivation using LiCl, reduced Egr1 and PTEN levels. Second, inhibition of β-catenin signaling by constitutively active GSK-3β resulted in increased expression of Egr1 and PTEN. Third, the involvement of β-catenin signaling was confirmed using constitutively active β-catenin and β-catenin shRNA. Finally, our PTEN promoter analysis showed that the Egr1-binding site is required for the suppression of PTEN transcription following the loss of E-cadherin, and is an important regulator of basal PTEN transcription. Interestingly, PTEN might also affect β-catenin signaling through suppression of PI3K/Akt signaling, suggesting a reciprocal relationship between the PTEN/PI3K/Akt and β-catenin signaling pathways (Persad et al., 2001). In addition, these two pathways have been shown to cooperate in tumor formation, as conditional inactivation of *Pten* and *Apc* in murine ovarian surface epithelium results in the formation of tumors that resemble human endometrioid ovarian carcinomas (Wu et al., 2007).

The role of PTEN in tumor growth has been extensively studied. Re-expression of PTEN in PTEN-deficient cells has been shown to induce growth suppression (Ramaswamy et al., 1999;Sun et al., 1999). In a variety of cells, overexpression of PTEN suppresses tumor cell growth by up-regulating $p27^{Kip1}$ and downregulating cyclin D1 (Furnari et al., 1998;Li and Sun, 1998;Persad et al., 2001;Radu et al., 2003;Weng et al., 2001;Weng et al., 1999). We have

demonstrated that overexpression of PTEN in SKOV-3 cells downregulates cyclin D1 and increases $p27^{kip1}$ protein levels. In addition, we observed similar results by inhibiting PI3K/Akt signaling with the PI3K inhibitor LY294002. Interestingly, LY294002 cannot completely abolished the reduction of $p27^{Kip1}$ in E-cadherin-depleted cells, suggesting that a PI3K/Akt-independent pathway may mediate the suppression of $p27^{Kip1}$ following E-cadherin loss. However, loss of β-catenin, either in SKOV-3 or SKOV-3/shEcad cells, results in downregulation of cyclin D1 and induction of $p27^{Kip1}$ protein levels. While our studies indicate that the alterations in cyclin D1 and $p27^{Kip1}$ protein levels following E-cadherin depletion are very likely due to the regulation of β-catenin signaling by PTEN/PI3K/Akt signaling, they also suggest a complex interplay between the pathways and/or the involvement of additional pathways.

E-cadherin is known to function in the density-dependent contact inhibition of cell growth. E-cadherin levels were increased in dense cultures and this was associated with increased levels of PTEN and reduced activation of the PI3K/Akt signaling pathway in OVCAR-3 and SKOV-3 ovarian cancer cells. Previous studies have suggested a link between reduced β-catenin signaling and up-regulation of E-cadherin (Conacci-Sorrell et al., 2003;Weng et al., 2002). These studies support our observation that β-catenin loss induces E-cadherin levels in sparse SKOV-3 ovarian cancer cells. Furthermore, we found that β-catenin depletion induces Egr1 and PTEN levels in sparse cultures, indicating that β-catenin signaling is important for the regulation of Egr1 and PTEN levels. We also demonstrated that disruption of E-cadherin-mediated cell-cell adhesion, by an inhibitory E-cadherin antibody, relocalizes β-catenin to nuclei and reduces Egr1 and PTEN levels in SKOV-3 ovarian cancer cells. These findings are in agreement with recent studies suggesting that cadherin-mediated cell-cell interactions can

regulate β-catenin/TCF signaling in colon cancer cells (Conacci-Sorrell et al., 2003;Maher et al., 2009). Taken together, our results demonstrate the importance of β-catenin signaling in the regulation of Egr1 and PTEN expression by E-cadherin-mediated cell-cell interactions.

In summary, we have demonstrated a role for E-cadherin in the transformed growth of ovarian cancer cells (Figure 4.8). The presence of E-cadherin acts to sequester β-catenin and maintain PTEN, thus permitting its tumor-suppressive function through the inhibition of PI3K/Akt signaling. Upon E-cadherin loss during tumor progression, the nuclear translocation and activation of β-catenin signaling leads to the suppression of Egr1, resulting in reduced PTEN transcription and activation of PI3K/Akt signaling. Under these conditions, the enhancement of PI3K/Akt signaling further stabilizes β-catenin signaling, via the phosphorylation-dependent inhibition of GSK-3β, and leads to increased transcription of oncogenic target genes that promote anchorage-independent growth (i.e. cyclin D1). Given that loss of E-cadherin is associated with ovarian cancer metastasis and peritoneal dissemination (Sawada et al., 2008;Veatch et al., 1994;Yuecheng et al., 2006), our data could have significant implications for tumor biology and cancer treatment. Specifically, the combined inhibition of PI3K/Akt and β-catenin signaling may block the transformed growth of such E-cadherin-deficient cells. Recent studies have shown that E-cadherin-deficient tumors are more resistant to treatment with epidermal growth factor receptor inhibitors (Black et al., 2008;Witta et al., 2006;Yauch et al., 2005). One possible explanation is that specific epidermal growth factor receptor inhibitors may fail to inhibit the growth of E-cadherin-deficient cells because β-catenin signaling could still promote the constitutive activation of PI3K/Akt signaling via inhibition of PTEN expression, thus promoting transformed growth.

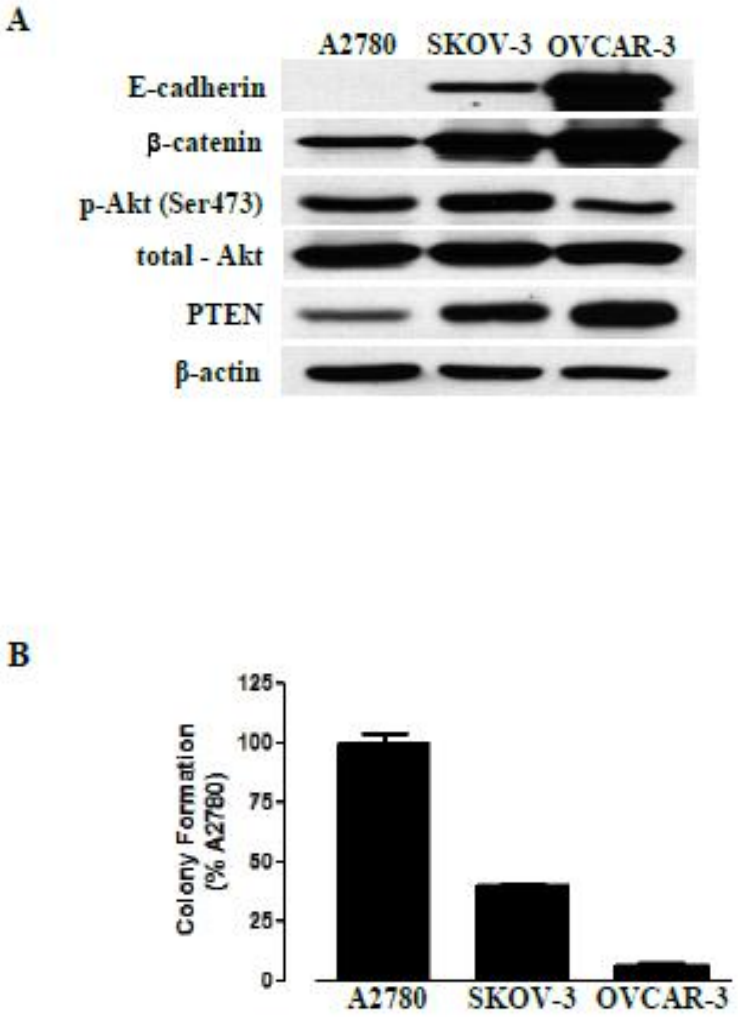


Figure 4.1

(A) Western blot analysis of E-cadherin β-catenin, phosphoylated and total Akt, phosphorylated, and β-actin levels in A2780, OVCAR-3 and SKOV-3 cells **(B)** A2780, SKOV-3 and OVCAR-3 cells were seeded in soft agar and analyzed for anchorage-independent growth. Results represent the mean ± SEM (n=3)

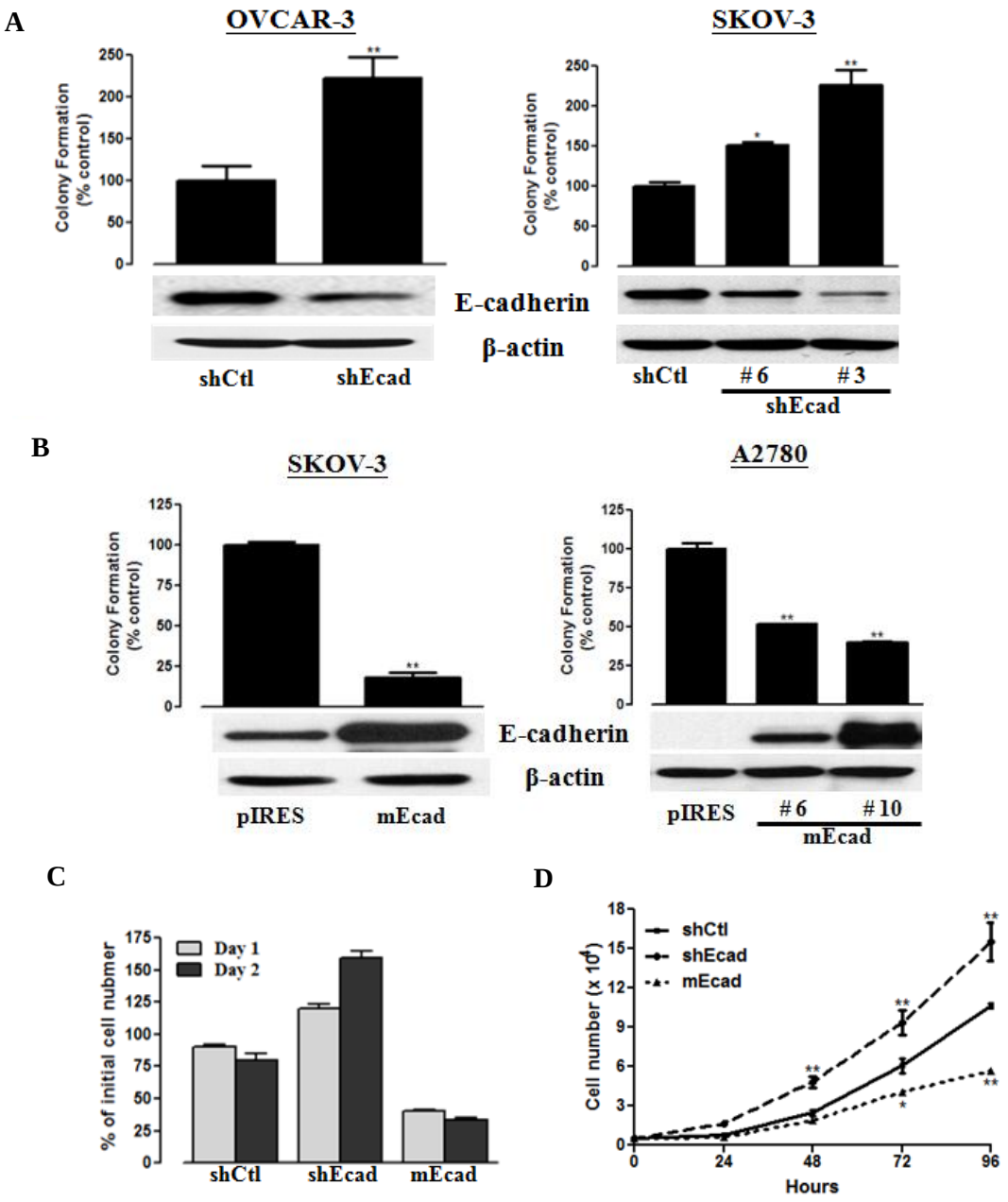


Figure 4.2 E-cadherin suppresses the growth of human ovarian cancer cells. **(A)** OVCAR-3 and SKOV-3 cells were stably transfected with scramble shRNA vector (shCtl) or E-cadherin shRNA vector (shEcad) and the ability of the cells to grow in soft agar was tested (*, $P < 0.05$; **, $P < 0.001$). Data represent the mean ± SEM (n=3). Representative immunoblot of E-cadherin protein levels in the various cell lines are shown in the lower panels. **(B)** A2780 and SKOV-3 cells were stably transfected with pIRES control vector (pIRES) or murine E-cadherin expression vector (mEcad) and the ability of the cells to grow in soft agar was tested. **(C)** Stably transfected SKOV-3 cells were placed in suspension and seeded in poly-HEMA coated plates, cultured for 1 or 2 days and viable cells were counted with Trypan blue. (mean ± SEM, n =3). **(D)** The ability of stably transfected SKOV-3 cell lines to grow on plastic was also determined. 12-well plates were seeded with 5 x 10^3 cells per well and cell growth was examined over time by cell counting. Results represent the mean ± SEM (n=3; *, $P < 0.05$; **, $P < 0.001$).

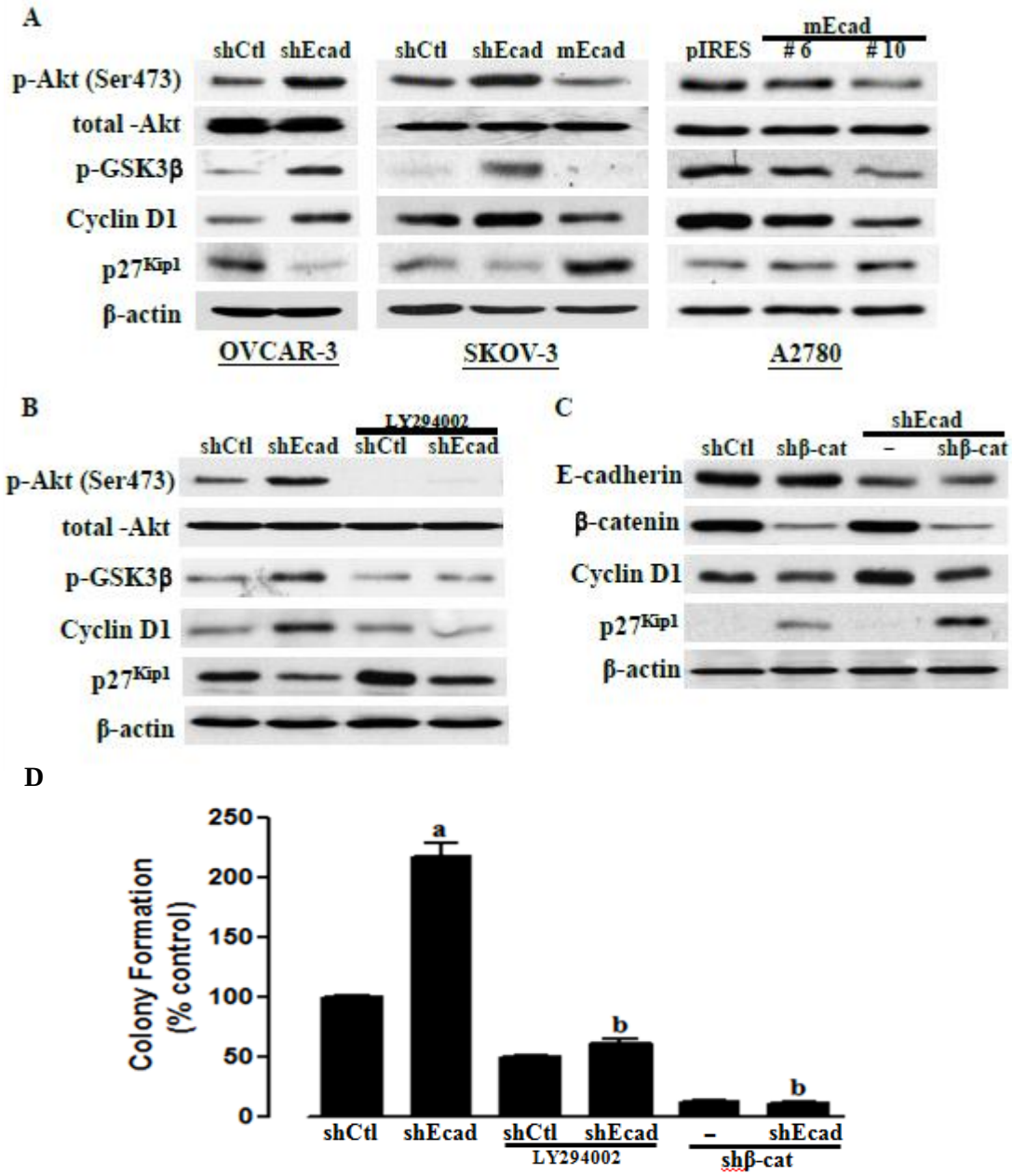


Figure 4.3 Loss of E-cadherin promotes anchorage-independent growth via the PI3K/Akt mediated β-catenin/TCF signaling pathway in human ovarian cancer cells (A) Western blot analysis of phosphoylated and total Akt, phosphorylated GSK3β, cyclin D1, $p27^{Kip1}$ and β-actin levels in A2780, OVCAR-3 and SKOV-3 cells (shCtl, shEcad or mEcad). **(B)** Stably transfected SKOV-3 cells were treated with DMSO or 10 μM LY294002 for 24 h and total cellular levels of phosphoylated and total Akt, phosphorylated GSK3β, cyclin D1, $p27^{Kip1}$ and β-actin were analyzed by Western blot. **(C)** Immunotblots showing E-cadherin, β-catenin, cyclin D1, $p27^{Kip1}$ and β-actin levels in shCtl, shβ-cat, shEcad and shEcad + shβ-cat cells. **(D)** Stably transfected SKOV-3 cells were seeded in soft agar in the presence of DMSO or 10μM LY294002 and analyzed for anchorage-independent growth. Results represent the mean ± SEM (n=3; a, $P < 0.001$, as compared with the SKOV-3/shCtl controls [DMSO]; b, $P < 0.001$, as compare with SKOV-3/shEcad controls).

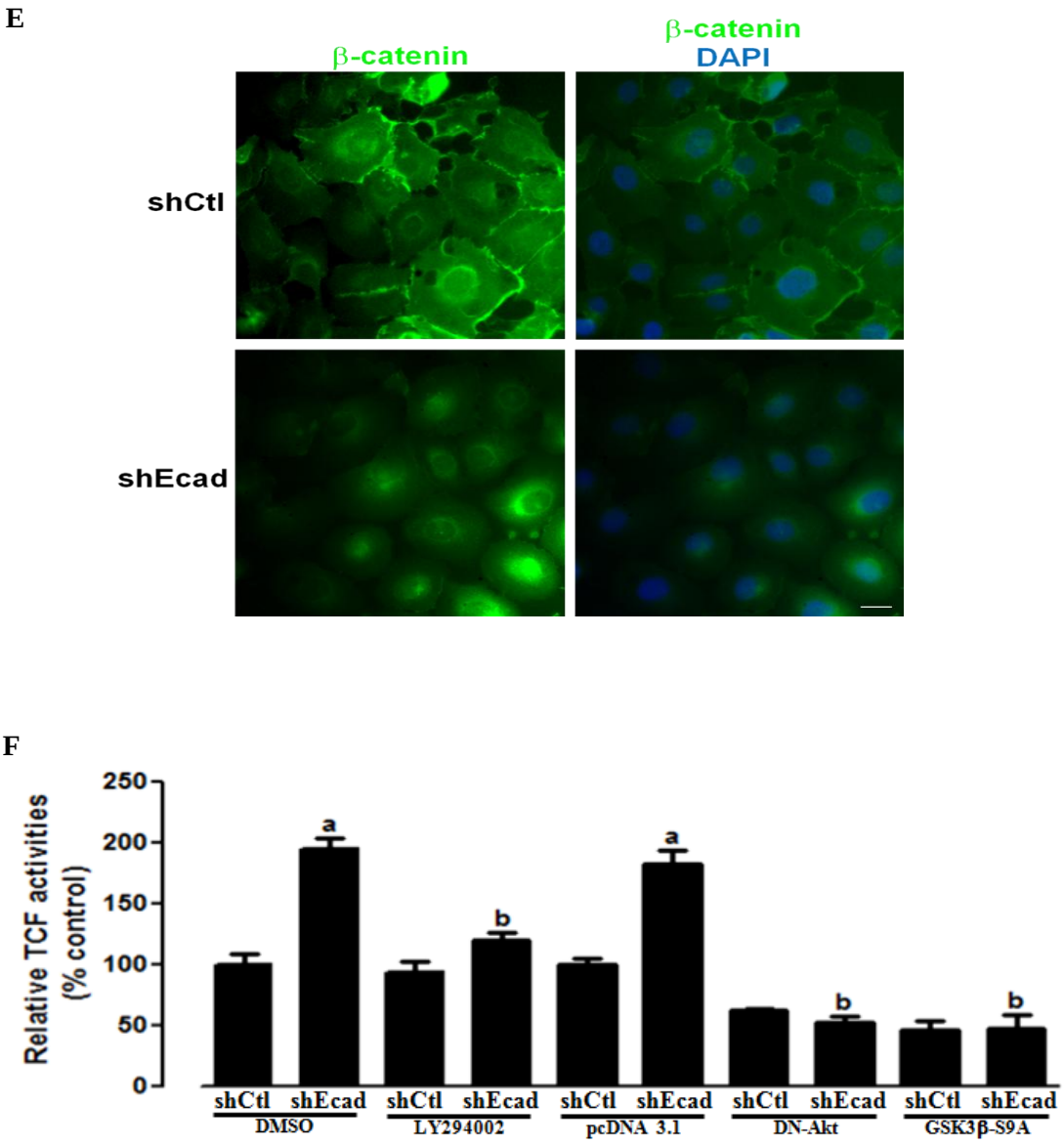


Figure 4.3 (E) SKOV-3 cells (shCtl or shEcad) were immunostained for β-catenin (green), cell nuclei were stained with DAPI (blue) and analyzed by fluorescence microscopy. Note the absence of β-catenin staining at cell-cell junctions and its nuclear localization in shEcad cells. Scale bar: 20 μm. **(F)** TCF activity was analyzed using the TOPFLASH and FOPFLASH luciferase reporters. Cells were transfected with either the TOPFLASH or FOPFLASH luciferase reporter, along with pcDNA 3.1, dominant negative Akt (DN-Akt), or constitutively active GSK3β (GSK3β-S9A). β-galactosidase vector was cotransfected for normalization of transfection efficiency. 10 μM LY294002 or DMSO was added for 24 h before harvesting the cells for the measurement of luciferase and β-galactosidase activities. Values are normalized luciferase activity (as described in the Materials and methods section) and are shown as mean ± SEM of three independent experiments performed in triplicate (a, $P < 0.001$, as compared with the SKOV-3/shCtl controls [DMSO or pcDNA3.1]; b, $P < 0.001$, as compare with SKOV-3/shEcad controls).

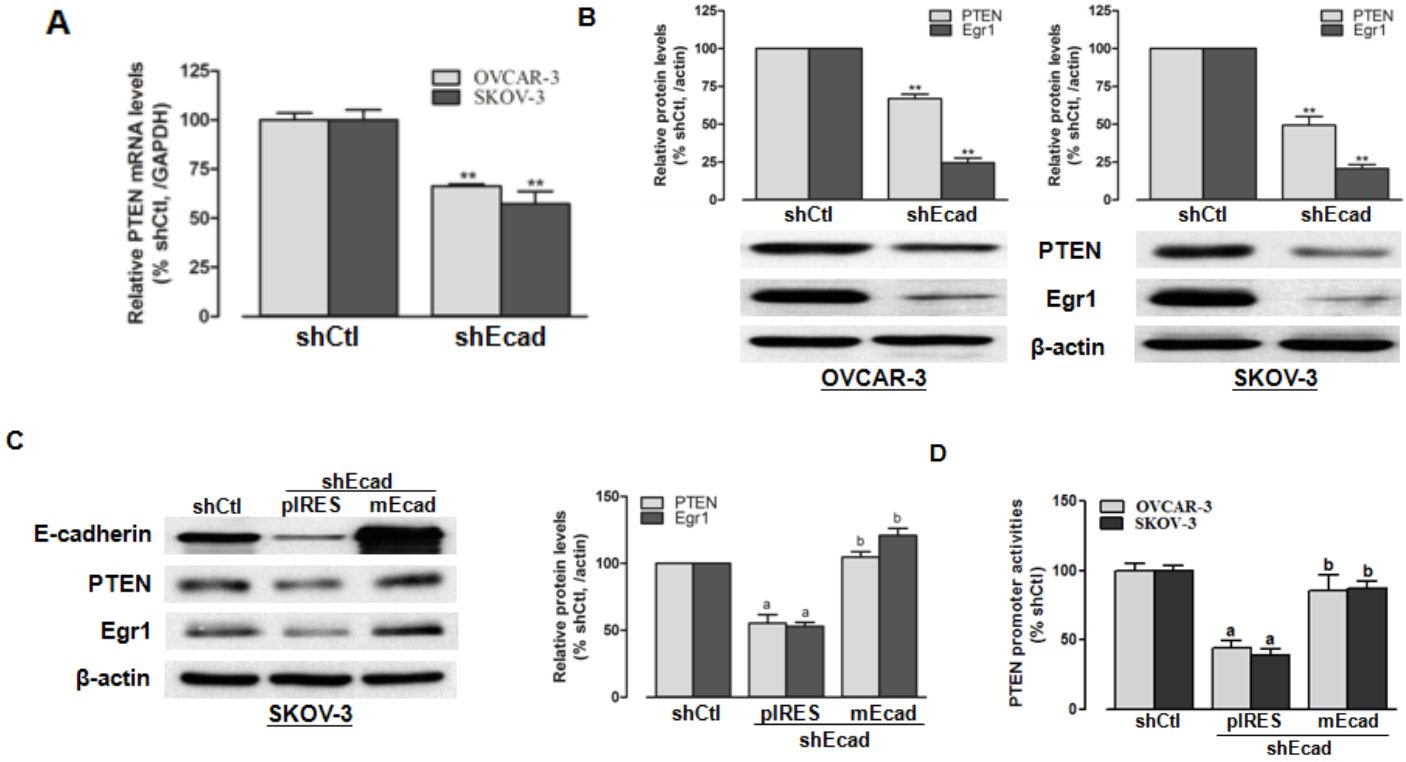


Figure 4.4 Loss of E-cadherin reduces PTEN mRNA and protein levels. (A) Relative PTEN mRNA levels in stably transfected cells were analyzed by real-time RT-PCR. Results represent the mean ± SEM (n=3; **, $P < 0.001$). **(B)** PTEN protein levels were analyzed by Western blot in stably transfected OVCAR-3 and SKOV-3 cells. Results represent the mean ± SEM (n=3; **, $P < 0.001$). **(C)** Stably transfected SKOV-3 cells were transient transfected with pIRES empty vector or murine E-cadherin expression vector (mEcad) for 24 h and subjected to immunoblotting for E-cadherin, PTEN and β-actin. . Results represent the mean ± SEM (n=3; a, $P < 0.001$, as compared with SKOV-3/shCtl control; b, $P < 0.001$, as compare with SKOV-3/shEcad controls [pIRES]). (**D**) Stably transfected OVCAR-3 and SKOV-3 cells were transient transfected with PTEN promoter construct and β-galactosidase plasmid. Twenty-four hours after transfection, cells were transfected with pIRES empty vector or murine E-cadherin expression vector (mEcad) for a further 24 h and subjected to Luciferase and β-galactosidase assays, and the luciferase activity of each sample was normalized by β-galactosidase activity. Results represent the mean ± SEM (n=3; a, $P < 0.001$, as compared with SKOV-3/shCtl control; b, $P < 0.001$, as compare with SKOV-3/shEcad controls [pIRES]).

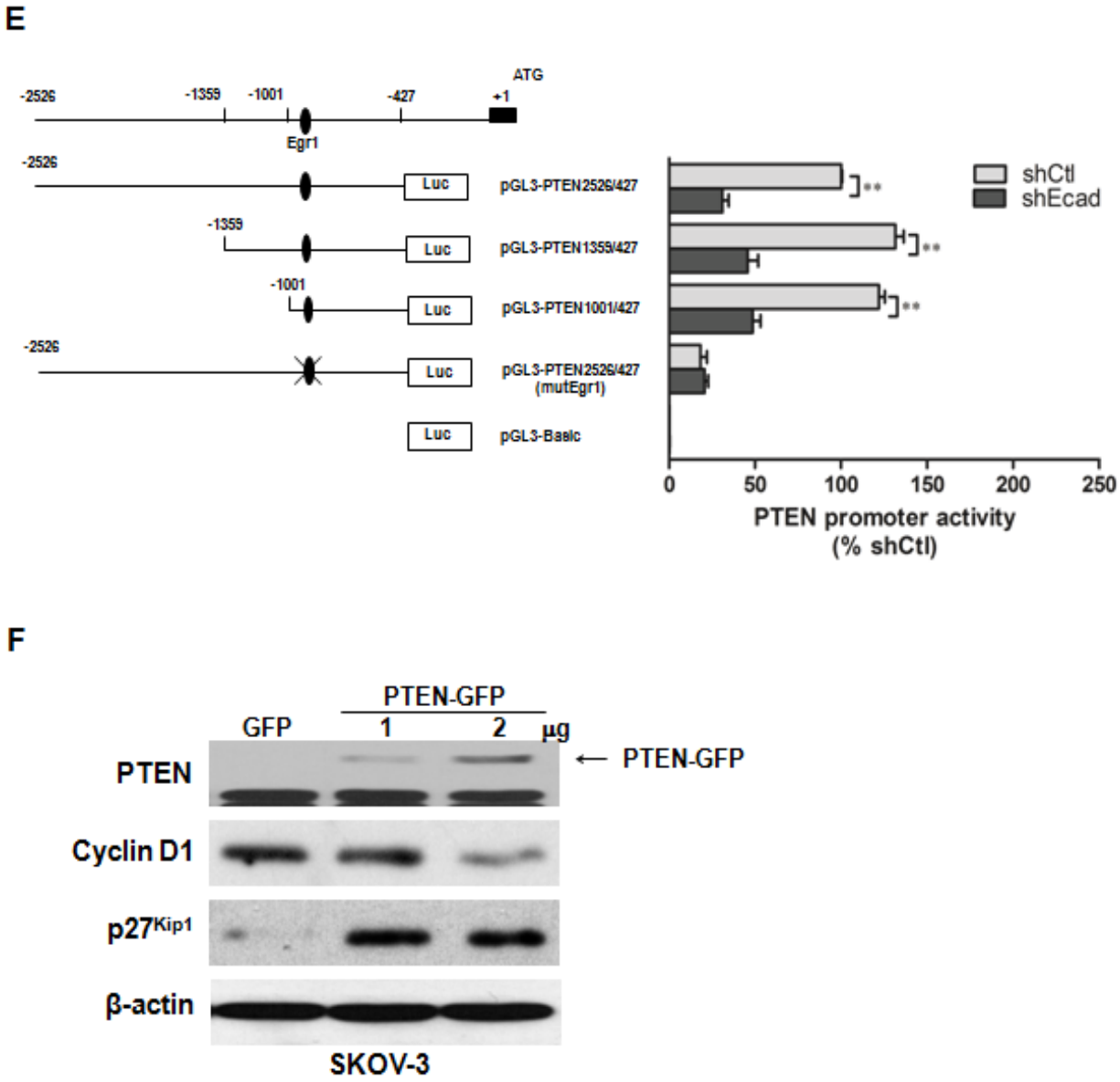


Figure 4.4 (E) Illustration of PTEN promoter reporters used for luciferase assay (left). Stably transfected SKOV-3 cells were transient transfected with pGL3-basic vector (pGL3-basic), truncated pGL3-PTEN promoters, Egr1 mutant promoter construct (mutEgr1) and β-galactosidase plasmid for 48 h and subjected to Luciferase and β-galactosidase assays, and the luciferase activity of each sample was normalized by β-galactosidase activity. Results represent the mean ± SEM (n=3; **, $P < 0.001$, as compared with SKOV-3/shCtl control). **(F)** SKOV-3 cells were transiently transfected with pcDNA-GFP (GFP) or pcDNA-PTEN-GFP (PTEN-GFP; 1-2 μg) for 24 h and subjected to immunoblotting for PTEN, cyclin D1, $p27^{Kip1}$, and β-actin. The total amount of plasmid DNA transfected in each group was balanced with pcDNA-GFP.

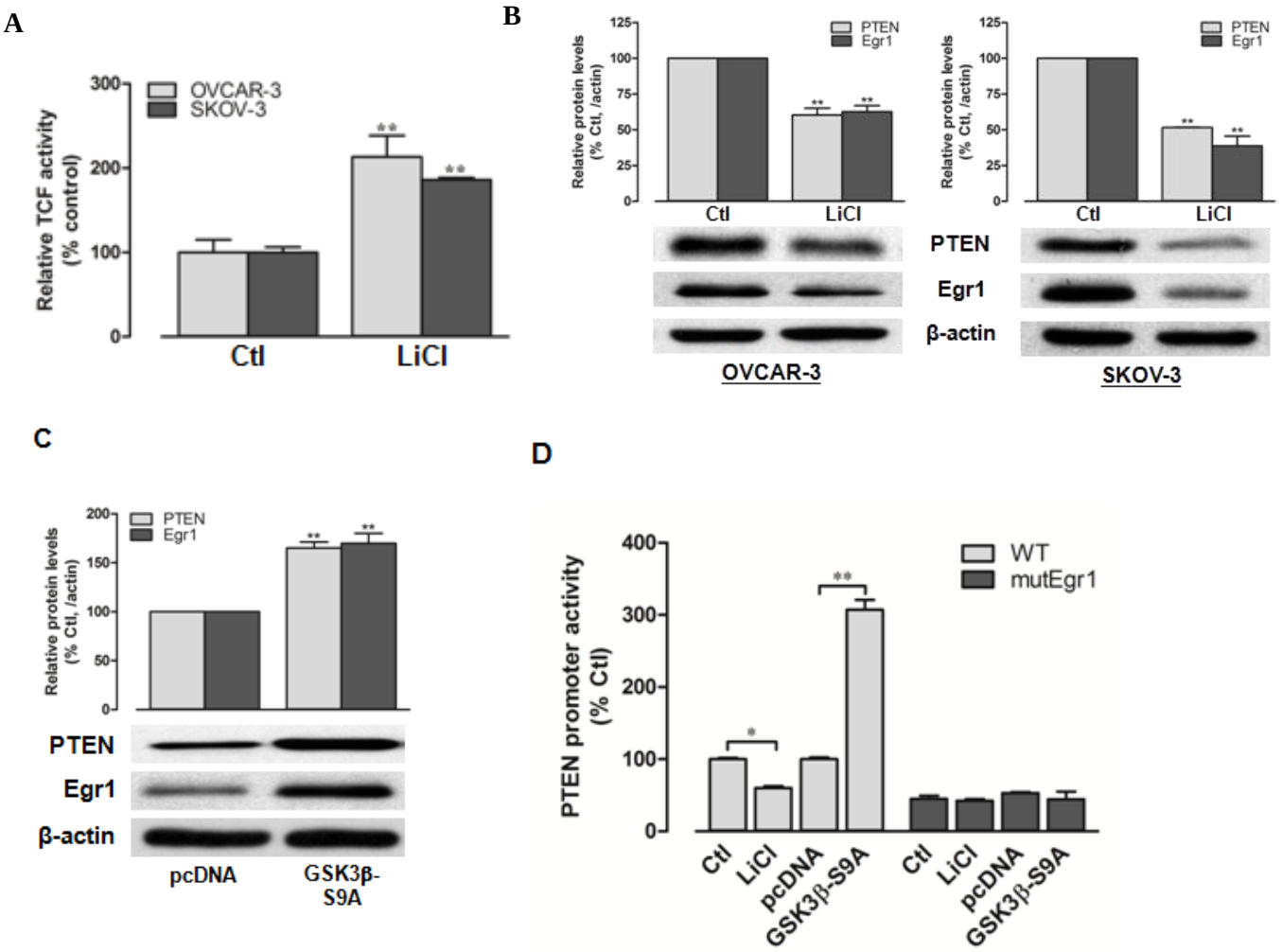


Figure 4.5 Loss of E-cadherin inhibits PTEN transcription via GSK3β inactivation (A) TCF activity was analyzed using the TOPFLASH and FOPFLASH luciferase reporters. Cells were transfected with either the TOPFLASH or FOPFLASH luciferase reporter, β-galactosidase vector was cotransfected for normalization of transfection efficiency. Twenty-four hours after transfection, 20mM LiCl was added for 24 h before harvesting the cells for the measurement of luciferase and β-galactosidase activities. Values are normalized luciferase activity (as described in the Materials and methods section) and are shown as mean ± SEM of three independent experiments performed in triplicate (**, $P < 0.001$). **(B)** OVCAR-3 and SKOV-3 cells were cultured for 24 h in the presence or absence of 20 mM LiCl and PTEN, Egr1 and β-actin protein levels were analyzed by Western blotting. **(C)** SKOV-3 cells were transiently transfected for 48 h with pcDNA 3.1 (pcDNA), or constitutively active GSK3β (GSK3β-S9A), and western blots of PTEN and β-actin protein levels were analyzed. Results represent the mean ± SEM (n=3; **, $P < 0.001$). **(D)** SKOV-3 cells were transient transfected with wild type PTEN (WT) or Egr1 mutant promoter construct (mutEgr1) and β-galactosidase plasmid. Twenty-four hours after transfection, cells were treated with 20mM LiCl, or transfected with pcDNA 3.1 (pcDNA), or constitutively active GSK3β (GSK3β-S9A) for a further 24 h and subjected to Luciferase and β-galactosidase assays, and the luciferase activity of each sample was normalized by β-galactosidase activity. Results represent the mean ± SEM (n=3; *, $P < 0.05$; **, $P < 0.001$).

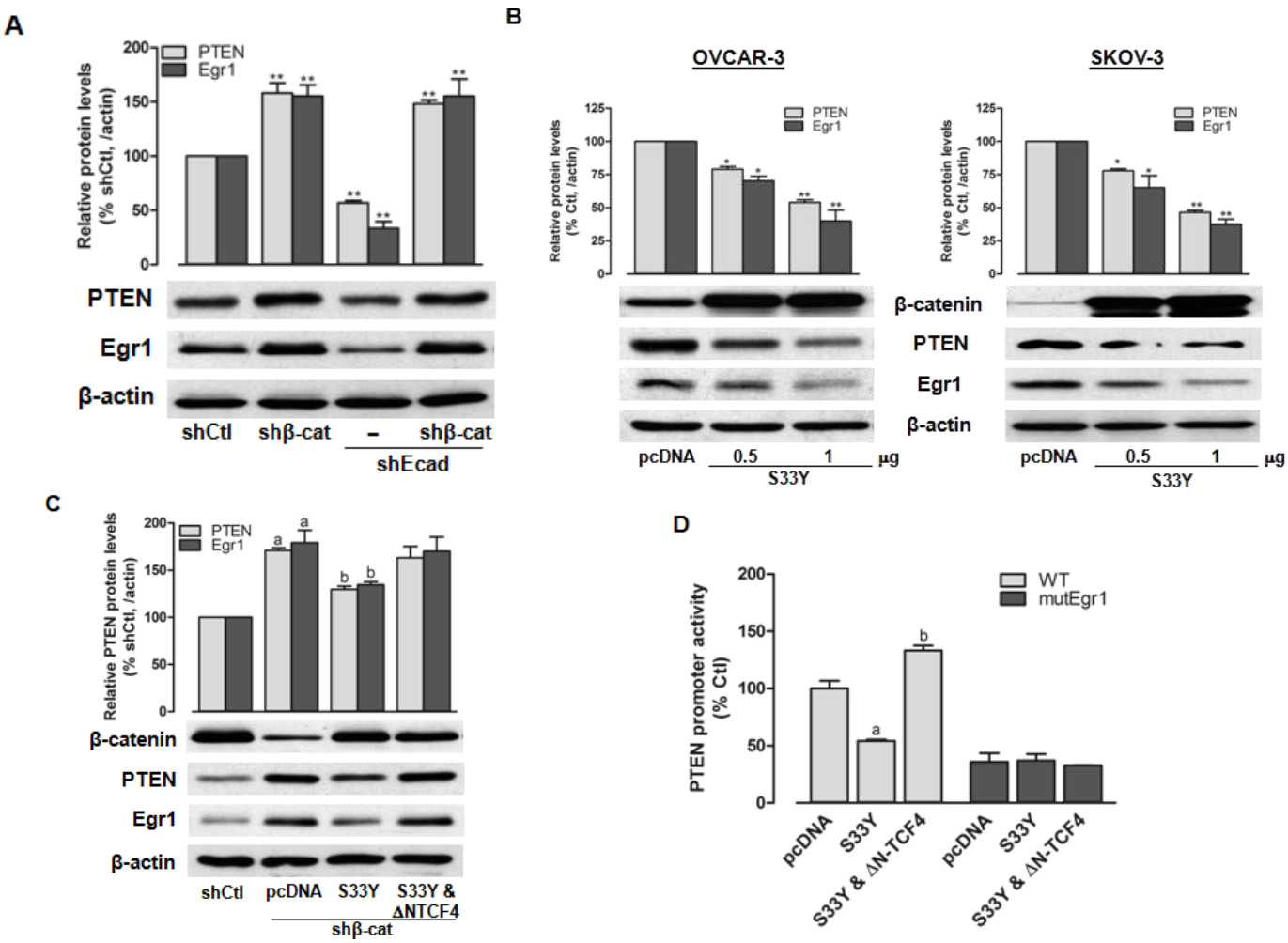


Figure 4.6 Loss of E-cadherin reduces PTEN levels through β-catenin/TCF signaling. (A) Protein levels of Egr1, PTEN and β-actin were determined in SKOV-3/shCtl, shβ-cat, shEcad, and shEcad + shβ-cat cells. **(B)** OVCAR-3 and SKOV-3 cells were transiently transfected with pcDNA 3.1 or constitutively active β-catenin (S33Y; 0.5-1 μg) for 24 h and subjected to immunoblotting for β-catenin, Egr1, PTEN, and β-actin. The total amount of plasmid DNA transfected in each group was balanced with pcDNA 3.1. Results represent the mean ± SEM (n=3; *, $P < 0.05$; **, $P< 0.001$). **(C)** β-catenin, PTEN and β-actin protein levels were examined in SKOV-3/shβ-cat cells transiently transfected with pcDNA 3.1 (pcDNA), constitutively active β-catenin (S33Y), or dominant negative TCF (ΔNTCF4). The total amount of plasmids transfected in each group was balanced with pcDNA 3.1. Results represent the mean ± SEM (n=3; a, $P < 0.001$, as compared with SKOV-3/shCtl control; b, $P < 0.05$, as compare with SKOV-3/shβ-cat controls [pcDNA]). **(D)** SKOV-3 cells were transient transfected with wild type PTEN (WT) or Egr1 mutant promoter construct (mutEgr1) and β-galactosidase plasmid. Twenty-four hours after transfection, cells were transfected with pcDNA 3.1 (pcDNA), constitutively active β-catenin (S33Y), or dominant negative TCF (ΔNTCF4) for a further 24 h and subjected to Luciferase and β-galactosidase assays, and the luciferase activity of each sample was normalized by β-galactosidase activity. The total amount of plasmids transfected in each group was balanced with pcDNA 3.1. Results represent the mean ± SEM (n=3; a, $P < 0.001$, as compared with pcDNA 3.1 control; b, $P < 0.001$, as compare with S33Y).

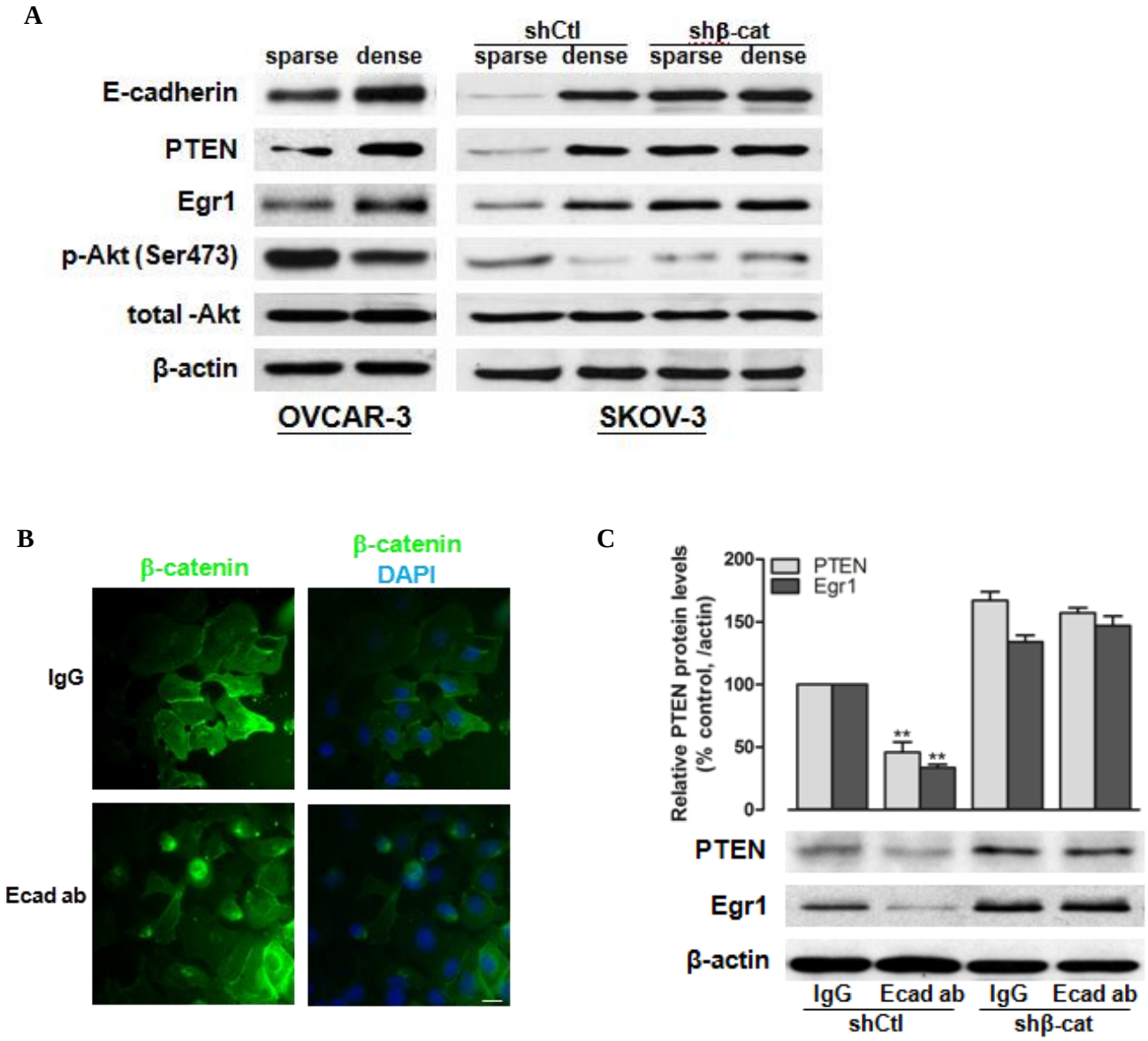
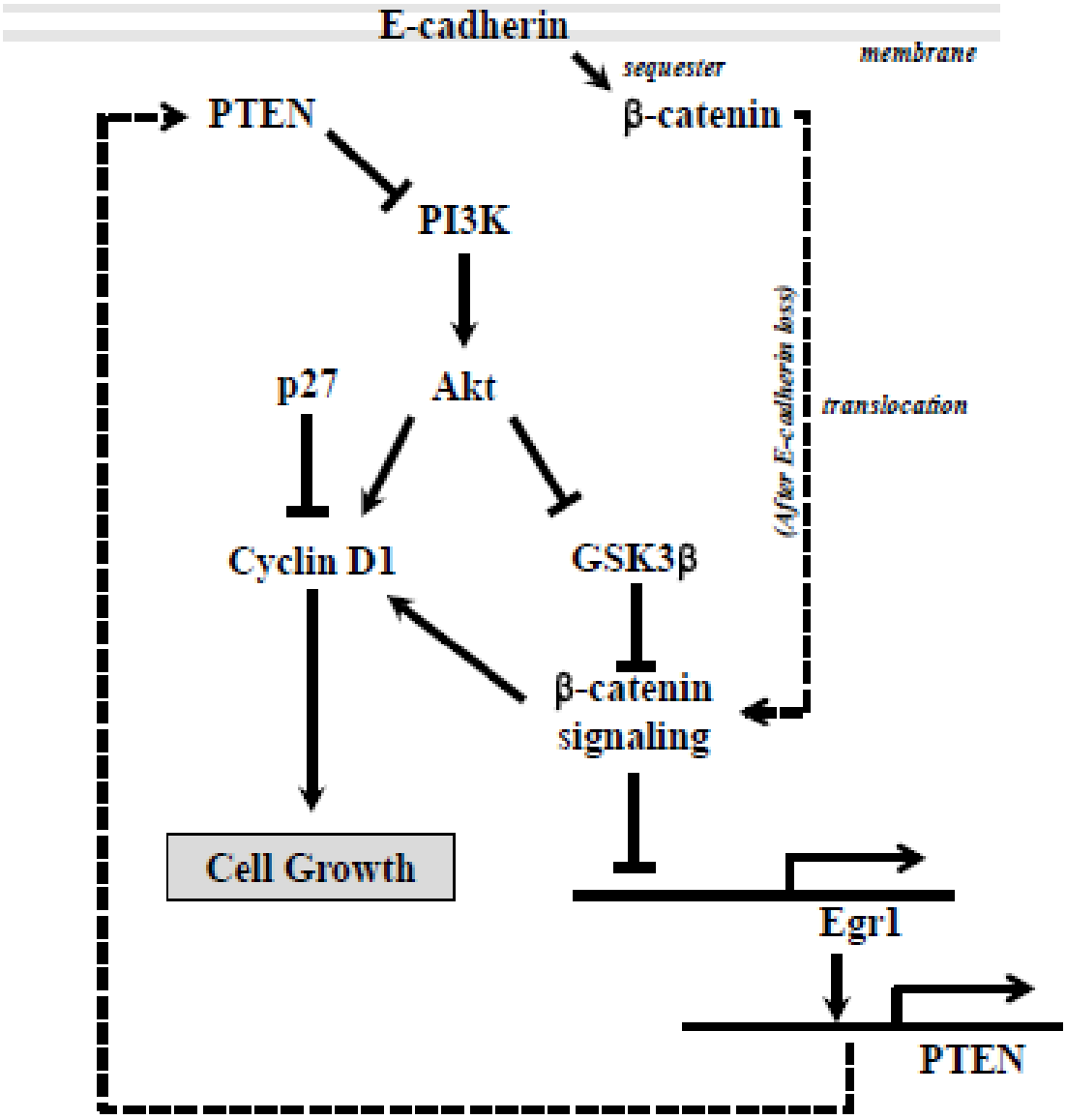


Figure 4.7 Regulation of PTEN levels by cell density and E-cadherin-cadherin interactions. **(A)** OVCAR-3, SKOV-3/shCtl, and SKOV-3/shβ-cat ovarian cancer cells were grown as sparse (6 x 10^3 cells/cm^2) or dense (6 x 10^4 cells/cm^2) cultures, and the levels of E-cadherin, PTEN, phosphoylated and total Akt, and β-actin were determined. **(B)** SKOV-3 cells were seeded as dense cultures in the presence of monoclonal mouse anti-E-cadherin antibody or control antibody, and the localization of β-catenin was examined by immunofluorescence microscopy. Scale bar: 20 μm **(C)** The levels of PTEN and β-actin were determined by Western blot analysis of lysates from SKOV-3 cells (shCtl or shβ-cat) incubated with anti-E-cadherin antibody or control antibody. Results represent the mean ± SEM (n=3; **, $P< 0.001$).



Figurer 4.8 Proposed model of E-cadherin action. The presence of E-cadherin inhibits PI3K/Akt signaling, reduces cyclin D1, and promotes increased levels of $p27^{Kip1}$, thus inhibiting cell growth. E-cadherin decreases the accumulation of β-catenin in the nucleus leading to increased levels of PTEN and resulting in reduced PI3K/Akt signaling. However upon E-cadherin loss during tumor progression, the accumulation of β-catenin in the nucleus leads to β-catenin/TCF transactivation and the suppression of PTEN levels, resulting in reduced negative regulation of the PI3K/Akt signaling pathway. Under these conditions, the activation of PI3K/Akt signaling further stabilizes β-catenin signaling by inhibiting GSK3β, thus leading to increased cell growth.

5. Conclusion and future directions

5.1 Overview

During tumor progression, acquisition of metastatic phenotypes is accompanied by the loss of E-cadherin function. Given the indispensable role of E-cadherin loss in promoting progression of many carcinomas, it is crucial to unravel the mechanisms that govern this process. Therefore, the studies described herein in order to define the molecular regulation of E-cadherin in ovarian cancer cells and to delineate the role of E-cadherin and its downstream transduction pathways in ovarian tumor progression.

First, I have shown the molecular mechanisms by which FGF2 (Chapter 2) and IGF1 (Chapter 3) down-regulate E-cadherin expression in ovarian cancer cells. I have presented evidence that several transcriptional repressors can mediate FGF2- and IGF1-induced E-cadherin suppression. I have also described the importance of their transduction signaling pathways in ovarian cancer cell growth and invasion. I have verified that the loss of E-cadherin can mediate FGF2-enhanced invasive potential of ovarian cancer cells. This work importantly relates the extracellular growth factors directly to ovarian cancer progression.

Chapter 4 of this thesis validates the idea that E-cadherin inhibits ovarian cancer cell growth. This work is a description of a novel mechanism showing the loss of E-cadherin is important to ovarian cancer cell growth. Here I correlated E-cadherin loss and activation of PI3K/Akt signaling with the ability of cells to grow in ovarian cancer. Finally, I have built upon these results in the subsequent chapter, which describes the molecular mechanisms of E-cadherin function in ovarian cancer cell invasion (Appendix). Taken together, this thesis provides more

insight into the regulation of E-cadherin expression in ovarian cancer, and provides a potential mechanism by which E-cadherin loss is associated to aggressive behaviour of ovarian cancer.

5.2 The roles of FGF2, IGF1 and E-cadherin in ovarian cancer development and metastasis

The ovary produces numerous cytokines, growth factors and hormones, and ovarian carcinomas express various receptors for these factors, which are potential candidates to associate with the ovarian cancer transformation and progression. Particularly, alterations of these factors production and receptor expression have been commonly found in ovarian tumors (Wong and Leung, 2007).

The observed association between higher levels of FGF2/IGF1 and cancer risk might arise because higher FGF2/IGF1 levels are involved in acceleration of the early events in tumor development. There is now increasing realization that tumor development needs stepwise accumulation of genetic changes that promote the progressive transformation of normal cells into highly aggressive malignancy. This process would be facilitated by mitogenic and antiapoptotic effects of these growth factors (Pollak et al., 2004). In the present studies it has been demonstrated that the PI3K/Akt/mTOR signaling pathway mediates the IGF1-induced ovarian cancer cell proliferation. Additionally, FGF2 is a well-known mitogen for ovarian cancer cell proliferation (Crickard et al., 1994;De Cecco et al., 2004;Di Blasio et al., 1993). In ovarian cancer patients, the overexpression of cyclins and cyclin-dependent kinase (CDK) correlate with poor prognosis (Reimer et al., 2006;Suh et al., 2008). It has been reported that both FGF2 and IGF1 can regulate cell cycle by stimulating cyclins (e.g. cyclin D1 and E1) and CDK, and suppressing CDK inhibitors (p16, p21, and p27) (Liu et al., 2001). Interestingly, IGF1 has been

shown to coordinate with FGF2 to promote cell cycle progression in oligodendrocyte progenitors (Frederick and Wood, 2004), thus implicating both FGF2 and IGF1 in playing important roles in early ovarian cancer development by promoting cell cycle progression and proliferation.

During ovarian tumor development, angiogenesis (formation of new vessels) is one of the early molecular events, and essential for tumor cell growth in size beyond 1 mm^3 (Folkman, 1995). This process is regulated by the balance between proangiogenic and antiangiogenic molecules in the tumor microenvironment. Vascular endothelial growth factor (VEGF), which is a potent regulator of angiogenesis (Folkman, 1992;Frumovitz and Sood, 2007), and other proangiogenic growth factors activate endothelial cells and endothelial progenitor cells from the bone marrow to form new blood vessels to provide oxygen and nutrients for tumor cells. During angiogenesis, FGF2 regulates the expression of various genes implicated in this process, including VEGF, collagenase, proteinase, and urokinase-type plasminogen activator (uPA) (Presta et al., 2005). IGF1 also promotes angiogenesis and migration in human endothelial cells (Shigematsu et al., 1999). In addition, IGF1 might indirectly play a key role in angiogenesis by inducing VEGF expression (Fukuda et al., 2002;Miele et al., 2000). This is further supported by a recent study demonstrating that siRNA targeting IGF1R inhibits angiogenesis in an ovarian cancer OVCAR-3 xenograft model (An et al., 2010). Taken together, FGF2 and IGF1, directly or indirectly, promote angiogenesis and the growth, differentiation, and migration of endothelium cells. Numerous FGFR tyrosin kinase inhibitors, which are in the early phases of clinical trials, have been developed by pharmaceutical companies. As the FGFR kinase domains have similar structure to VEGF receptor (VEGFR), many FGFR inhibitors also inhibit VEGFR. The dual inhibition, targeting two progangiogenic growth factors, has the obvious potential benefit of targeting angiogenesis and tumor cell proliferation (Turner and Grose, 2010).

At the later stage during the development of ovarian cancer, primary cancer cells acquire the ability to detach from neighbouring tissues, and begin to invade adjacent tissues, and metastasize to colonize metastatic tumors on distal sites (Bast, Jr. et al., 2009). Many growth factors are involved in this process, including VEGF, EGF, HGF, TGFβ, tumor necrosis factor α (TNFα) and lysophosphatidic acid (LPA), through the activation of the MAPK/ERK, PI3K/Akt/p70S6K, and STAT3 signaling pathways (Bast, Jr. et al., 2009). In agreement with this notion, the present study has shown the importance of the MAPK/ERK and PI3k/Akt/mTOR signaling pathways in FGF2-induced cell invasion in ovarian cancer. p70S6K, which is a downstream mediator of mTOR signaling is known to promote ovarian cancer cell migration (Ip et al., 2011). Thus, p70S6K might be involved in the FGF2-induced cell invasion. Further studies are required to elucidate the role of p70S6K in FGF2-induced ovarian cancer cell invasion. Indeed, IGF1 can also promote ovarian cancer invasion via these two signaling pathways (Shen et al., 2004).

In addition, adhesion proteins also play major role in the tumor invasion and metastasis, including calcium-dependent cadherin families, as well as integrins, which link cells to extracellular matrix (ECM) substrates (Hanahan and Weinberg, 2000). Loss of E-cadherin function is common in a majority of carcinomas. In ovarian carcinomas, loss of E-cadherin has been correlated with high tumor grade, peritoneal dissemination and poor prognosis (Cho et al., 2006;Daraï et al., 1997), implicating the tumor suppressive E-cadherin function in ovarian tumor progression. Given that elevated levels of FGF2/IGF1 have been observed in the late stage of ovarian cancer, the present study further supports the inhibiting effect of both FGF2 and IGF1 on E-cadherin expression via up-regulation of transcription repressors, Snail, Slug and ZEB1. Interestingly, the expression of Snail and Slug was elevated stepwise in ovarian benign,

borderline and malignant tumors (Yoshida et al., 2009), indicating that high expression of Snail/Slug family members may be involved in late-stage ovarian carcinoma metastatic progression (Elloul et al., 2005;Imai et al., 2003). A recent study has also reported the important role of Snail/Slug-induced E-cadherin loss in ovarian cancer cell radioresistance and chemoresistance by antagonizing p53-mediated apoptosis and gaining a stem-like phenotype (Kurrey et al., 2009). Furthermore, a recent study has demonstrated that Gab2 (a scaffolding adaptor protein) induces migration and invasion, as well as E-cadherin down-regulation via the induction of PI3K/Akt-dependent ZEB1 expression (Wang et al., 2011). The present study has demonstrated that the loss of E-cadherin itself is sufficient to promote invasiveness via induction of PI3K/Akt/mTOR signaling and Rho GTPase activity in ovarian cancer. Taken together with the present work, loss of E-cadherin function is critical for ovarian cancer cell invasion and metastasis, suggesting that E-cadherin and its transcription repressors may have the potential to serve as a screening marker of ovarian carcinomas.

Unlike many other carcinomas, dissemination of ovarian carcinoma (but not ovarian serous carcinomas) via the blood vessel is unusual. The dissemination can be either achieved by direct extension to neighbouring organs, or transportation of exfoliated tumor cells throughout the peritoneal cavity by normal peritoneal fluid. The metastasized ovarian cancer is commonly accompanied with ascites formation (Naora and Montell, 2005), in which ovarian carcinoma cells often exist as multicellular aggregates (Burleson et al., 2004). To metastasize, ovarian cancer cells must first detach from primary tumor site. Detachment of normal epithelial cells from ECM triggers apoptosis, a phenomenon termed anoikis (Chiarugi and Giannoni, 2008;Simpson et al., 2008). However, most metastatic cancer cells have acquired resistance to anoikis which is critical for tumor cell dissemination and survival in peritoneal fluid. Our results

have shown that loss of E-cadherin function promotes the anchorage-independent cell growth in ovarian cancer cells, emphasizing the important role of E-cadherin-depletion in anoikis regulation during tumor cell dissemination. On the other hand, recent studies have demonstrated that FGF2 and IGF1 repress anoikis in human pluripotent stem cells and melanoma cells, respectively. Thus, high levels of growth factors in ascetic fluid from ovarian cancer patients, such as FGF2 and IGF1, might also contribute to the anoikis resistance. Future studies are required to investigate the role of these factors in anoikis resistance in ovarian cancer.

Finally, ovarian cancer metastasis to the surface of peritoneal cavity requires the attachment of cancer cells to the mesothelial cells. This process is mediated by adhesion proteins such as integrin. Integrins are heterodimeric receptors that consist of α and β subunits, and function as cell-ECM adhesion proteins. Integrins mediate signals in response to ECM to regulate various cellular functions, including cell adhesion, proliferation, differentiation, migration and invasion (Desgrosellier and Cheresh, 2010). Increasing evidence has indicated that integrins promote cell migration and invasion during tumor progression (Guo and Giancotti, 2004;Mitra and Schlaepfer, 2006), which requires the invading and metastasizing cancer cells to adapt to a novel extracellular matrix environment. Therefore, metastatic cells promote invasion by the conversion of integrins from those that favour the ECM present in primary tumor site to other integrins that preferentially attach to the degraded stromal molecules generated by extracellular proteases (Lukashev and Werb, 1998;Varner and Cheresh, 1996). In ovarian cancer, overexpression of integrin β1 has been found to be associated with higher clinical stage and poor survival (Dong et al., 2010;Li et al., 2010). Up-regulation of integrin β1 expression promotes ovarian cancer cell migration and MMP-dependent cell invasion (Casey and Skubitz, 2000;Ellerbroek et al., 1999). Moreover, the integrin β1 subunit has been demonstrated to

facilitate ovarian cancer cell adhesion to peritoneal mesothelial cells and to increase peritoneal metastasis (Lessan et al., 1999;Slack-Davis et al., 2009). Our unpublished results have also shown that integrin β1 mediates EGF-enhanced invasiveness in ovarian cancer cells (Lau et al., unpunished data). In addition, a recent study has shown that loss of E-cadherin induces metastasis via integrin α5 in ovarian cancer *in vivo* model (Sawada et al., 2008). It has been demonstrated that FGF2 stimulates integrin α5 and β1 subunits in NIH 3T3 fibroblast cells (Klein et al., 1996), whereas IGF1 elevates integrin α3 and β1 subunits in breast carcinoma cells (Barazi et al., 2002). It will be interesting to study the roles of integrins in FGF2/IGF1-induced ovarian cancer progression in the future.

In summary, ovarian tumorigenesis is a complicated biological process at the cellular level, involving regulation of cell survival, proliferation, angiogenesis, invasion, and metastasis. Many growth factors and their downstream signaling pathways, such as MAPK/ERK and PI3K/Akt, promote tumor progression via regulation of various genes, including E-cadherin, involved in both cell survival as well as metastasis. In the future, the identification of genes and/or signaling pathways involved in the control of ovarian tumorigenesis provides new insight to discover potential therapeutic targets and interventions.

5.3 Future perspective

Research during the past few decades on the origin of ovarian carcinoma has predominantly focused on their "normal" counterpart tissue, OSE. Therefore, many of the previous studies suggesting ovarian cancer-related genes have been based on a comparison in their expression levels between ovarian carcinoma and OSE. Hence, due to lack of E-cadherin

expression in OSE, E-cadherin has been found to "over-express" in ovarian carcinoma, and has been described to play a key role in early neoplastic transformation in ovarian cancer patients (Reddy et al., 2005). However, recent morphological and molecular genetic studies have revealed that the different ovarian carcinoma subtypes display distinct molecular genetic signatures, which are concordant with those of normal tissues they resemble and show little linkage to OSE (Kurman and Shih, 2010). Thus, the expression profiles of serous carcinoma resemble the distal normal fallopian tube epithelium, whereas the genes expressed in endometrioid and clear cell carcinomas show similarities to those expressed in endometrial epithelium. Interestingly, the gene expression profiles of mucinous tumors have been found to resemble normal colonic epithelium (Marquez et al., 2005). In the future, analysis of E-cadherin gene expression in ovarian carcinomas should take into account their histological subtypes being studied and the data compared to more appropriate normal tissues (Kurman and Shih, 2010). Moreover, future experiments regarding the early molecular events in the development of different ovarian carcinoma subtypes should concentrate on the appropriate normal tissues.

5.4 Limitations of studies

One limitation of cell lines is that they have often been cultured in an artificial environment which may not accurately reflect the peritoneal microenvironment of the initial tumor *in vivo*. The gene expression profiles (such as different receptors and signaling mediators) may be different in *in vitro*-based and *in vivo* systems, leading to different response to their environmental stimuli. Therefore, three-dimensional cell culture systems that mimic the *in vivo* environment will provide more important insights into ovarian tumor biology (Kenny et al.,

2008). Additionally, mouse xenograft model can be used to examine the role of E-cadherin-deficient in the metastasis of ovarian cancer.

Second, the cell lines used in this study were established several decades ago. The origins of these cell lines are poorly defined. Moreover, due to the genomic instability of the cancer cells, cell lines, through serial passage, may accumulate additional genetic mutations that are not present in the initial tumor. For example, cell lines with some genetic lesions (e.g., *KRAS*/*BRAF* mutation) may response differently to E-cadherin loss. Recently, there is an increase in knowledge of the histologic subtypes of ovarian cancer, the field would benefit from the establishment of a new panel of ovarian cancer cell lines or primary cultures with different representative histologic and molecular subtypes that reflect the various originating cells, as well as underlying genomic events driving each subtype (Schauer et al., 2011). Better understanding of molecular pathways in the context of specific cancer subtypes will be critical in future therapeutic direction, be useful for new therapeutic strategies such as personalized therapy, and eventually will have implications for cancer prevention.

5.5 Conclusions

The present study has further emphasized the potential role of FGF2 and IGF1 in promoting ovarian tumor progression via down-regulation of E-cadherin. Moreover, this study increases our understanding of the impact of E-cadherin on the tumor growth and invasiveness, provides insight into ovarian tumor progression, and provides new approaches for the evaluation of new therapeutic strategies. Thus, E-cadherin functions as a tumor suppressor gene in ovarian cancer.

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Appendix. E-cadherin inhibits ovarian cancer cell invasion by suppressing PI3K/Akt/mTOR signaling and Rho GTPase activation

A.1 Introduction

Epithelial ovarian cancer (EOC), which comprises 90% of all ovarian malignancies, is the most common and lethal form of gynecological cancer in developed countries (Jemal et al., 2005), the death rate for this disease has not changed much in the last 50 years.

E-cadherin functions as a cell-cell adhesion molecule and tumor suppressor that is silenced in many cancers, and the loss of E-cadherin function or expression is a common event in tumor progression (Nollet et al., 1999;Yap, 1998). E-cadherin is known to suppress tumor cell invasion, and the re-expression of E-cadherin in E-cadherin-deficient carcinomas results in a less invasive, less aggressive phenotype (Gottardi et al., 2001;St Croix et al., 1998;Yanagisawa and Anastasiadis, 2006), while the loss of E-cadherin expression is associated with ovarian cancer metastasis, peritoneal dissemination, and poor prognosis (Daraï et al., 1997;Hudson et al., 2008;Sawada et al., 2008;Veatch et al., 1994;Yuecheng et al., 2006). The loss of E-cadherin function can be achieved by the mutation of the E-cadherin gene (Hajra and Fearon, 2002), the hypermethylation of the E-cadherin promoter (Graff et al., 1995;Hennig et al., 1995), and the transcriptional repression of E-cadherin (Batlle et al., 2000;Cano et al., 2000;Comijn et al., 2001;Hajra et al., 2002). Several transcription factors have been identified to suppress E-cadherin including Snail, Slug, Twist and ZEB1 via their interaction with the E-box binding site in the E-cadherin promoter (Batlle et al., 2000;Bolos et al., 2003;Cano et al., 2000;Grooteclaes and Frisch, 2000;Peinado et al., 2007).

Rho family of GTPases, which includes Rho, Cdc42 and Rac1, is essential for regulation of the cytoskeleton and cell adhesion (Bourne et al., 1991). As with other GTPases, the members

of this family shift between an GTP-bound (active form) and GDP-bound (an inactive form). Rho proteins are important for cell adhesion, cell migration and are involved in cell polarity and wound healing (Fukata et al., 1999;Gumbiner, 1996;Takeichi, 1995).

Previous studies have demonstrated that loss of E-cadherin expression in human ovarian cancer cells resulted in the activation of PI3K/Akt signaling pathway (Lau et al., 2011). In the present study, we demonstrate that E-cadherin-depletion enhances ovarian cancer cell invasion via the PI3K/Akt/mTOR signaling. Furthermore, elevated Cdc42 and Rac1 activation mediates the E-cadherin-depletion-induced invasion.

A.2 Materials and methods

A.2.1 Materials

E-cadherin antibody was purchased from BD Biosciences (San Jose, CA). Akt, phospho-Akt (Ser 473), p70S6K and phospho-p70S6K (Thr389) antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA). LY294002 and rapamycin were purchased from Calbiochem (San Diego, CA). The β-actin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG antibodies were purchased from Bio-Rad Laboratories (Hercules, CA).

A.2.2 Cell culture and transfections

Human ovarian cancer cell lines (A2780, OVCAR-3 and SKOV-3) were purchased from the American Type Culture Collection (ATCC, Manassas, VA), and their use was approved by the University of British Columbia Clinical Screening Committee for Research and Other Studies Involving Human Subjects. Cells were cultured in Medium 199:MCDB 105 (1:1; Sigma-

Aldrich Corp., St. Louis, MO) containing 10 % fetal bovine serum (FBS; Hyclone Laboratories Ltd., Logan, UT), 100 U/ml penicillin G and 100 g/ml streptomycin (Life Technologies, Inc., Rockville, MD) in a humidified atmosphere of 5 % CO_2- 95 % air at 37°C. The cells were passaged with 0.06 % trypsin (1:250)/ 0.01 % EDTA in Mg^{2+} /Ca^{2+} - free HBSS at confluence.

A.2.3 Plasmid constructs

pLKO.1 expression vectors containing short hairpin sequences targeting human E-cadherin (shEcad, GCAGAAATTATTGGGCTCTTT), and a pLKO.1 scramble shRNA vector were generously provided by Dr. Robert A. Weinberg (Onder et al., 2008). Stable, pooled populations were then generated under puromycin selection (2 μg/ml, Sigma), and clones were isolated by limiting dilution.

An IRES expression vector (Clonetech, Palo Alto, CA) containing full-length wild-type mouse E-cadherin (mEcad) cDNA was a kind gift from Dr. Antonino Passaniti (Sasaki et al., 2000). Stable, pooled populations were then generated under G418 selection (400 μg/ml, Invitrogen Canada, Burlington, ON, Canada), and clones were isolated by limiting dilution.

N17Cdc42 (DN-Cdc42) and N17Rac1 (DN-Rac1) vectors were obtained from UMR cDNA Resource Center (Rolla, MO, USA).

All transfections were carried out using Lipofectamin 2000 Reagent (Invitrogen) according to the manufacturer's protocol.

A.2.4 Western blot analysis

Cells were harvested in lysis buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 1 mM EDTA, 0.1% SDS] that contained a protease inhibitor cocktail

(Sigma), and protein concentrations were determined using the Bradford protein assay (Bio-Rad Laboratories, Inc., Hercules, CA). Protein (40 µg) was electrophoresed on 7.5 % SDS-polyacrylamide gels, transferred to nitrocellulose membranes (Amersham Bioscience), and incubated with specific primary antibodies at 4 ^{o}C overnight. After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h and visualized with enhanced chemiluminescent substrate (Thermo Fisher Scientific Inc, CA).

A.2.5 Invasion assay

Twenty-four-well transwell inserts with an 8-µm pore coated with 1mg/ml Matrigel (50 µl/well; BD sciences, Mississauga, ON, Canada) were used to assess cell invasion. Trypsinized cells (1×10^5) in 0.1% FBS medium, with or without FGF-2, were seeded in triplicate in the upper chamber. 1% FBS medium was placed in the lower wells. The chambers were incubated for 24 h at 37 ^{o}C in a 5% CO_2 atmosphere. Cells that did not penetrate the filter were wiped off, and invaded cells on the lower surface of the filter were fixed with ice-cold methanol and stained with 0.5% crystal violet. Results are presented as the mean number of invaded cells of five fields (at 100x magnification) ± SEM from three independent experiments.

A.2.6 Cdc42 and Rac1 pull down assays

Cdc42/Rac1 pull-down assays were performed using, respectively, Cdc42/Rac1 Activation Assay Kit (Upstate Biotechnology, Lake Placid, NY, USA), according to the manufacturer's instructions. Briefly, cells were seeded the day before the analysis, washed with ice cold PBS, and lysed with cell lysis buffer. Equal amounts of protein from cell extracts were used for pull-down assays and incubated for 1 h at 4 ^{o}C with 10 µl of PAKPBD protein agarose

beads. Pellets were washed three times with 0.5 ml of washing buffer and eluted with 20 μl of Laemmli buffer. Samples were analyzed by SDS–polyacrylamide gel electrophoresis. Western blots were then carried out using mouse anti-Cdc42 polyclonal antibodies or mouse anti-Rac1 monoclonal antibody (Upstate Biotechonology). The total levels of RhoA, Cdc42 and Rac1were measured by western blot performed on the cell extract.

A.2.7 Data analysis

All the experiments were performed at least three times. All values are expressed as mean ± SEM. Data were analyzed by Student's *t*-test or one-way ANOVA followed by Tukey's *post hoc* test using GraphPad Prism 5 (GraphPad Software, San Diego, CA). $P < 0.05$ was considered statistically significant.

A.3 Results and discussion

A.3.1 Loss of E-cadherin induces cell invasion in human ovarian cancer cells

As the mechanism by which E-cadherin promotes suppression of invasiveness is still unclear, we examined the effects of E-cadherin depletion cells on the invasive capacity of ovarian cancer cells, a Boyden chamber assay using a Matrigel-coated invasion chamber has been used. Our results demonstrate that knockdown of endogenous E-cadherin increased cell invasion in OVCAR-3 and SKOV-3 cells (Figure A.1A). In contrast, the expression of exogenous murine E-cadherin (mEcad) suppressed cell invasion in A2780 and SKOV-3 cells (Figure A.1B).

A.3.2 Loss of E-cadherin induces cell invasion via the PI3K/Akt mediated mTOR signaling pathway in human ovarian cancer cells

My result shows that the loss of E-cadherin induces the PI3K/Akt signaling which has been implicated in promotion of tumor cell cell motility, invasion and metastatic potential (Franke, 2008). Depletion of endogenous E-cadherin increases basal phosphorylation of Akt (Figure A.2A). PI3K/Akt signaling is known to lead to activating downstream mTOR signaling pathway. We hypothesized that E-cadherin-depletion-mediated activation of PI3K/Akt signaling may lead to the activation of the mTOR signaling pathway. Consistent with this hypothesis, we found that E-cadherin depletion increased the phosphorylation of the mTOR downstream molecule, p70S6K, in both SKOV-3 and OVCAR-3 cells (Figure A.2A). We used LY294002 to confirm that the mTOR pathway is a downstream substrate of PI3K/Akt signaling induced by the loss of E-cadherin. Treatment with LY294002 completely abolished the increase in phosphorylated p70S6K observed in E-cadherin-depleted OVCAR-3 and SKOV-3 cells (Figure A.2B), thus suggesting a role for PI3K/Akt in mTOR signaling activation following the loss of E-cadherin. In contrast, expression of murine E-cadherin suppressed Akt and p70S6K phosphorylation (Figure A.2A). These data show that the Akt/mTOR signaling pathway is activated in E-cadherin-depleted human ovarian cancer cells. To determine if the PI3K/Akt/mTOR signaling pathway was involved in the loss of E-cadherin-induced invasion, we treated SKOV-3 control cells (shCtl) and E-cadherin-depleted cells (shEcad) with the PI3K inhibitor LY294002 or the mTOR-specific inhibitor rapamycin. Treatment with LY294002 or rapamycin abolished the shEcad-mediated increases in cell invasion (Figure A.2C). These data strongly indicate that loss of E-cadherin promotes ovarian cancer cell invasion by activating the PI3K/Akt/mTOR signaling pathway.

A.3.3 Loss of E-cadherin stimulates the activity of Rho family proteins

The Rho small GTPases family, Cdc42, Rac1 and RhoA, are molecular switches that modulate the organization and dynamics of the actin cytoskeleton (Raftopoulou and Hall, 2004b;Ridley et al., 2003b), which is critical for cell motility (Pantaloni et al., 2001). Several lines of evidence indicate that E-cadherin can regulate Rho GTPase (Asnaghi et al., 2010;Noren et al., 2001). To determine if E-cadherin regulates Rho GTPase activities, we measured Cdc42 and Rac1 activation using a pull-down assay that specifically recognized the active GTP-bound form in stably transfected OVCAR-3, SKOV-3 and A2780 cells. As shown in Figure 5.3A, E-cadherin-depleted OVCAR-3 cells were found to induce the activation of Cdc42 and Rac1. In contrast, the activation of Cdc42 and Rac1 were reduced in E-cadherin-overexpressing SKOV-3 and A2780 cells. To investigate if E-cadherin-depletion induced cell invasion through Cdc42 and Rac1, we performed the invasion assay with dominant negative mutants of Cdc42 and Rac1. Expression of N17Cdc42 (DN-Cdc42) or N17Rac1 (DN-Rac1) was shown to clearly abolished the E-cadherin-depletion-induced cell invasion in OVCAR-3 and SKOV-3 cells (Figure A.3).

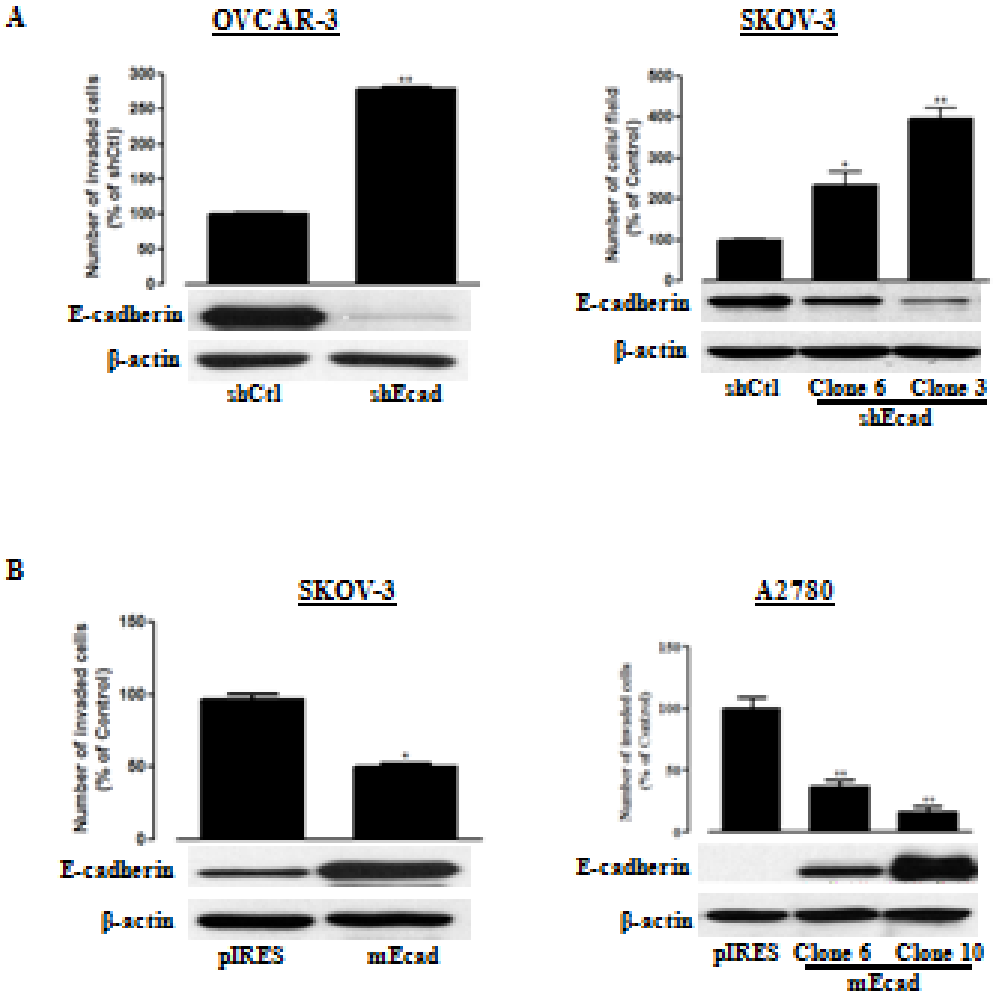


Figure A.1 E-cadherin suppresses the invasion of human ovarian cancer cells. **(A)** OVCAR-3 and SKOV-3 cells were stably transfected with scramble shRNA vector (shCtl) or E-cadherin shRNA vector (shEcad) and the ability of the cell invasion was tested (*, $P < 0.05$; **, $P < 0.001$). Data represent the mean ± SEM (n=3). Representative immunoblot of E-cadherin protein levels in the various cell lines are shown in the lower panels. **(B)** A2780 and SKOV-3 cells were stably transfected with pIRES control vector (pIRES) or murine E-cadherin expression vector (mEcad) and the ability of the cell invasion was tested.

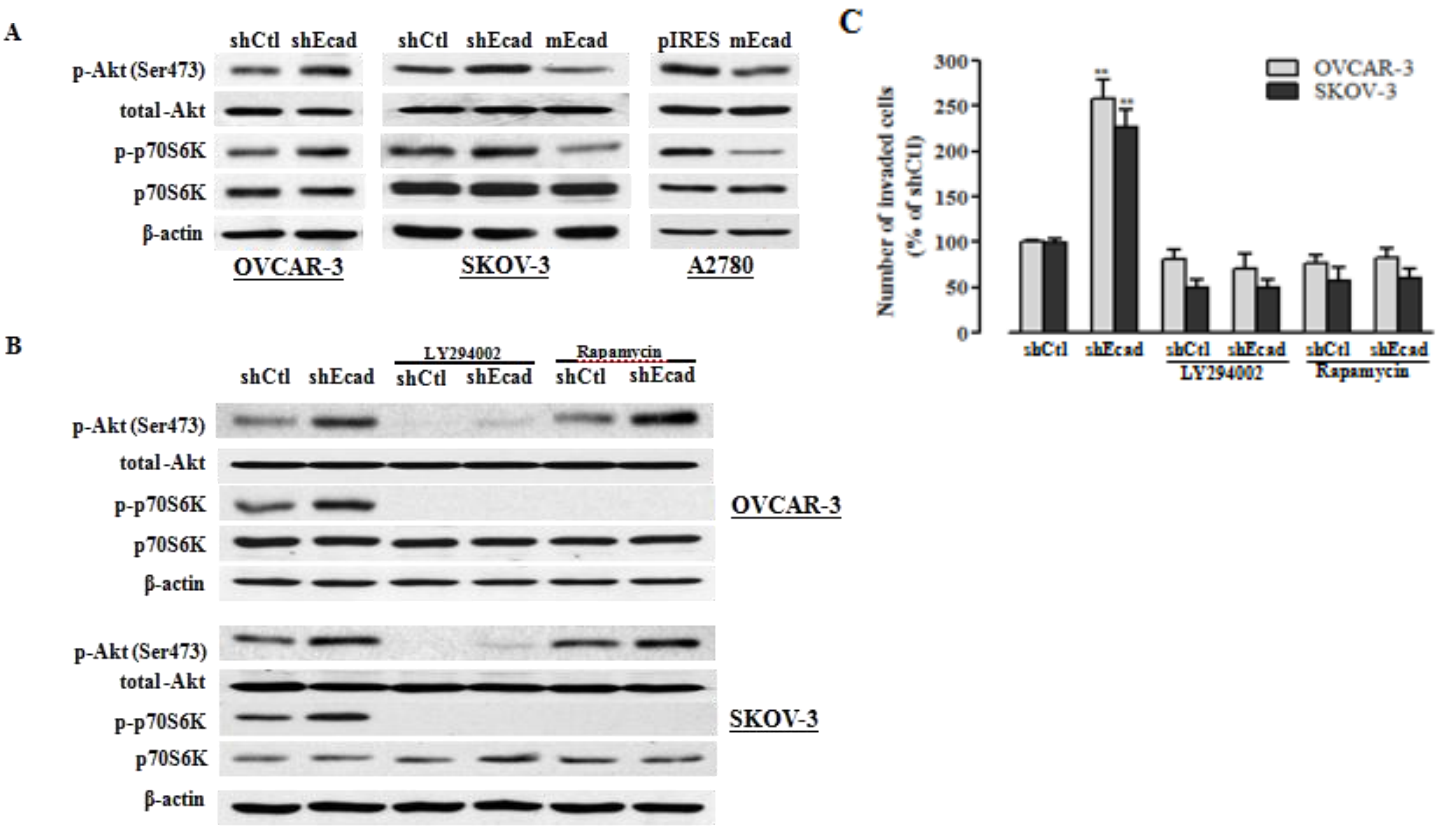
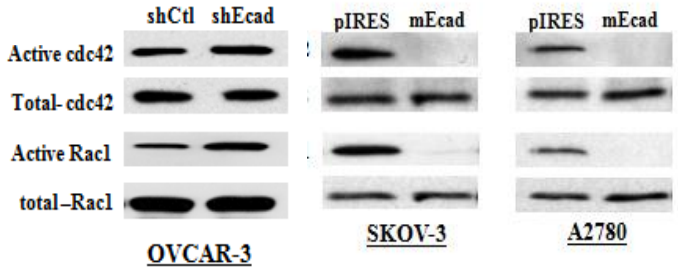


Figure A.2 Loss of E-cadherin induces tumor cell invasion via the PI3K/Akt/mTOR signaling pathway in human ovarian cancer cells. **(A)** Western blot analysis of phosphoylated and total Akt, phosphorylated and total p70S6K and β-actin levels in A2780, OVCAR-3 and SKOV-3 cells (shCtl, shEcad or mEcad). **(B)** Stably transfected OVCAR-3 and SKOV-3 cells were treated with DMSO or LY294002 (10 μM) or rapamycin (20 nM) for 30 min and total cellular levels of phosphoylated and total Akt, phosphorylated and total p70S6K and β-actin were analyzed by Western blot. **(C)** Stably transfected SKOV-3 cells were seeded in Matrigel-coated invasion chambers in the presence of DMSO or LY294002 (10 μM) or rapamycin (20 nM) for 24 h and analyzed for invasion assay. Results represent the mean ± SEM (n=3; **, $P < 0.001$).

A



B

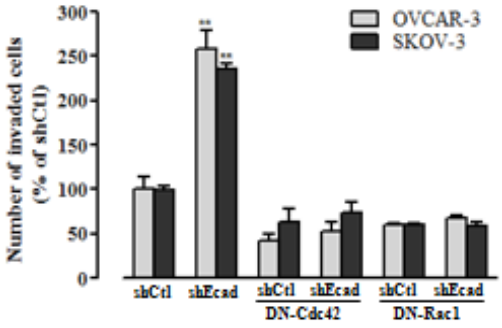


Figure A.3 Loss of E-cadherin induces tumor cell invasion via activation of Cdc42 and Rac1 in human ovarian cancer cells. **(A)** Cdc42-GTP and Rac1-GTP pull-down assays were performed in stably transfected OVCAR-3, SKOV-3 and A2780 cells and levels of Cdc42 and Rac1 were analyzed with Western blot. **(B)** Stably transfected OVCAR-3 and SKOV-3 cells were transient transfected with pcDNA3.1 (empty vector), N17Cdc42 (DN-Cdc42), and N17Rac1 (DN-Rac1) for 48h. After transfection, the trypsinized cells were seeded in Matrigel-coated transwell inserts for 24h. Results represent the mean ± SEM (n=3; **, $P < 0.001$).