# ROLE OF E-CADHERIN IN THE SEROUS BORDERLINE OVARIAN TUMOR AND LOW-GRADE SEROUS OVARIAN CARCINOMA CELL INVASION

by

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## Abstract

E-cadherin is a membrane glycoprotein located at cell adherens junctions. A switch from E-cadherin to N-cadherin expression has been considered a hallmark of the epithelial-mesenchymal transition (EMT), which is primarily due to the up-regulation of the transcription factors Snail, Slug, Twist and ZEB1. Epithelial ovarian cancer cells with low E-cadherin expression are more invasive, and the absence of E-cadherin expression in ovarian cancer is associated with poor prognosis and survival. Serous borderline ovarian tumors (SBOT) are slow-growing, non-invasive ovarian epithelial neoplasms. SBOT are considered distinct entities that give rise to invasive low-grade serous carcinomas (LGSC), which have a relatively poor prognosis and are unrelated to high-grade serous carcinomas (HGSC). The mechanisms underlying the progression of non-invasive SBOT to invasive LGSC are not understood.

We have established short-term cultures of SBOT cells from tumor biopsies and have shown that inactivation of p53, Rb and/or PP2A by the SV40 large T (LT) and small T (ST) antigens allows SBOT cells to acquire characteristics associated with neoplastic progression, including increased cell motility, invasion and EMT. However, the overexpression of N-cadherin does not induce cell invasion in SBOT cells. In this study, using loss- and gain-of-function approaches, we show that p53 acts as a tumor suppressor in the regulation of SBOT and LGSC cell invasion by regulating E-cadherin expression through PI3K/Akt-mediated transcriptional and epigenetic machineries.

In high-grade ovarian cancer cultures, it has been shown that epidermal growth factor (EGF) and transforming growth factor-beta (TGF-β) induce cell invasion by activating the EMT. However, the effects of EGF and TGF-β on SBOT and LGSC cell invasion remain unknown. We show that EGF induces SBOT cell invasion by activating the EMT. In addition, our results suggest that there are EMT-independent mechanisms that mediate EGF-induced

LGSC cell invasion. Interestingly, we show a dual function for TGF-β in which it induces invasion in SBOT cells by activating the EMT and promotes apoptosis in LGSC cells. Overall, this study demonstrates that the loss of E-cadherin expression in SBOT may play an important role in the transition to invasive LGSC.

## Preface

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

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

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## List of Abbreviations

ANOVA: Analysis of variance

APC: Adenomatous polyposis coli

APST: Atypical proliferative serous tumors

CDK: Cyclin-dependent kinase

CGH: Comparative genomic hybridization

DNMT: DNA methyltransferases

ECM: Extracellular matrix

EGF: Epidermal growth factor

EGR1: Early growth response 1

EMT: Epithelial-mesenchymal transition

FAK: Focal adhesion kinase

FGF: Fibroblast growth factor

FIGO: International Federation of Gynecology and Obstetrics

GSK3β: Glycogen synthase kinase 3-beta

HGSC: Low-grade serous ovarian carcinomas

HGF: Hepatocyte growth factor

hTERT: Human telomerase reverse transcriptase

IGF: Insulin-like growth factor

ILK: Integrin-linked kinase

LGSC: Low-grade serous ovarian carcinomas

LMP: Serous low malignant potential tumors

MAPK: Mitogen-activated protein kinase

MEF: Mouse embryonic fibroblast

mFISH: Multicolor fluorescent *in situ* hybridization

MMP: Matrix metalloproteinases

MPSC: Micropapillary serous carcinomas

OSE: Ovarian surface epithelium

PDGF: Platelet-derived growth factor

PI3K: Phosphatidylinositol 3-kinase

PP2A: Protein phosphatase 2A

Rb: Retinoblastoma protein

RTK: Receptor tyrosine kinases

RT-qPCR: Reverse transcription quantitative real-time PCR

SBOT: Serous borderline ovarian tumors

SNP: Single nucleotide polymorphism

SV40: Simian virus 40

TGF-β: Transforming growth factor-beta

TIMP: Tissue inhibitor of metalloproteinases

tPA: Tissue-type plasminogen activator

uPA: Urokinase-type plasminogen activator

WHO: World Health Organization

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# Chapter 1. Introduction

## 1.1 Ovarian cancer

### 1.1.1 Overview

Ovarian cancer is the most lethal gynecological cancer and is the 5th leading cause of cancer death, with an estimated 225,500 new cases and 140,200 deaths occurring worldwide in 2011 (1). Ovarian cancer is a disease that is neither common nor rare. Compared to 1 in 8 for breast cancer, the lifetime risk of a woman developing ovarian cancer is 1 in 70 (1.4%) (2-4). Despite the 5-year survival rate significantly increasing from 37% in the 1970s to approximately 46% in the current decade due to a vast amount of clinical and laboratory research, improved surgical techniques and the availability of treatments with empirically optimized combinations of chemotherapeutic agents, the overall cure rate for ovarian cancer remains approximately 30% (5, 6).

Ovarian cancer is often called the "silent killer". Because of the absence of early symptoms, approximately 70% of patients are diagnosed at an advanced stage (FIGO stages III and IV) and have disease that has already spread beyond the ovaries (7, 8). Due to the residual microscopic and macroscopic peritoneal implants, most patients cannot be cured by surgery, thereby resulting in a major cause of lethality in ovarian cancer. However, if the disease is detected at an early stage (FIGO stage I), when the tumor is limited to the ovary, the 5-year survival rate increases dramatically to 90% (9, 10). Given that metastasis is the critical challenge in the clinical management of ovarian cancer, a better understanding of the molecular mechanisms that regulate cancer cell motility and invasion will significantly improve the outcomes of this devastating disease.

**1.1.2 Types of ovarian cancer**

Both benign and malignant ovarian tumors can arise from different ovarian cell types. Approximately 3% of ovarian cancers are germ cell tumors that are derived from the egg-producing cells of the ovary, such as dysgerminomas and choriocarcinomas. Sex cord-stromal tumors, such as granulosa cell tumors and thecomas, are derived from the connective tissue of the ovary and account for 7% of ovarian cancers (11, 12). Both germ cell tumors and sex cord-stromal tumors are rare. Germ cell tumors are most common in children and young adults, whereas sex cord stromal tumors can appear in any age group and are found most often in older women (13, 14). Epithelial ovarian cancer is the most common type of ovarian cancer; it comprises approximately 90% of all ovarian cancers and is thought to arise from the OSE or its inclusion cysts (15, 16).

**1.1.3 Subtypes of epithelial ovarian cancer**

Epithelial ovarian cancer is heterogeneous at both the genetic and molecular levels (17). According to World Health Organization (WHO) criteria, epithelial ovarian cancers are divided into several histologic subtypes based on tumor morphology (Table 1.1) (18). The four major subtypes are serous, endometrioid, clear cell and mucinous. Among these major subtypes, serous epithelial ovarian cancers are the most common (19). There are other subtypes, including transitional, undifferentiated and malignant mixed. Unlike most cancers that undergo de-differentiation during transformation, epithelial ovarian cancers develop from simple flattened OSE into four distinct main subtypes that resemble the epithelium of the fallopian tube (serous), endometrium (endometrioid), glycogen-filled vaginal rests (clear cell) and mucin-secreting endocervical glands (mucinous) (12, 20).

Recently, based on the histological grade and molecular genetic analysis, epithelial

ovarian cancers have been divided into two groups, designated Type I and Type II cancers (Table 1.2) (21-23). Type I cancers include low-grade serous (LGSC), low-grade endometrioid, clear cell and mucinous subtypes. They grow slowly and are often diagnosed in early stages (FIGO stages I and II). Type I cancers have more frequent *KRAS*, *BRAF*, *PTEN*, PI3K catalytic subunit-α (*PIK3CA*) and β-catenin (*CTNNB1*) mutations, whereas mutations in *p53* are rare (21-25). Type II cancers include high-grade serous (HGSC), high-grade endometrioid and undifferentiated subtypes. They grow aggressively and usually present at late stages (FIGO stages III and IV). Type II cancers have a high frequency of *p53* (50-80%), *BRCA1* and *BRCA2* mutations, as well as genomic instability (21-25).

There is increasing evidence that the different histologic subtypes of ovarian cancer are different diseases and have different natural behavior, etiology and prognosis. At present, it is believed that these differences should be reflected in both clinical and laboratory research designs and eventually in the management of ovarian cancer.


**1.1.4 Source of epithelial ovarian cancer**

Traditionally, it was accepted that epithelial ovarian cancers arise from the OSE and the lining of ovarian epithelial inclusion cysts (21, 26). The strong evidence that supports this origin of epithelial ovarian cancers is based on developmental history, animal models and the potential stemness of the OSE (26-31). Recently, a new concept has been proposed that suggests that high-grade serous ovarian cancers, which are the most common and lethal of all ovarian epithelial cancers, may arise from the epithelium of the fimbriae of the fallopian tubes and then metastasize to the surface of the ovary (32). Nevertheless, serous borderline tumors, low-grade serous carcinomas and mucinous cancers originate from the OSE (22, 32, 33). Piek et al. were first to discover tubal intraepithelial carcinomas in the fimbriae of

prophylactic salpingo-oophorectomy specimens in patients with a *BRCA* mutation (34). Later, studies showing the morphologic resemblance of the tubal intraepithelial carcinomas to HGSC, and *p53* mutation, which is characteristic of HGSC, provided the strongest evidence that HGSC arise from the epithelium of the fimbriae (35, 36).

## 1.2 Serous ovarian carcinomas

### 1.2.1 Overview

Serous ovarian carcinomas are the most prevalent type of carcinoma and constitute the majority (70%) of epithelial ovarian carcinomas. Although recent molecular and genetic studies have highlighted the differences between the various histologic subtypes, to date, only serous ovarian carcinomas have been studied extensively. Histopathologically, serous ovarian carcinomas display a papillary structure, slit-like spaces and solid sheets of tumor cells with nuclear atypia and high mitotic activity (19). Like most cancers, it was proposed that serous ovarian carcinomas progressed from OSE to benign serous ovarian cystadenomas, to serous borderline ovarian tumors (SBOT; also known as LMP: serous low malignant potential tumors; or APST: atypical proliferative serous tumors), to low-grade serous ovarian carcinomas (LGSC) and finally to high-grade serous ovarian carcinomas (HGSC). Over the past decade, due to advanced technologies such as oligonucleotide microarrays, array comparative genomic hybridization (array CGH) and single nucleotide polymorphism (SNP) arrays, large-scale molecular genetic studies have argued against this outdated concept and have revealed two distinct pathways leading to the development of low-grade versus high-grade serous ovarian carcinomas (Figure 1.1) (22).

**1.2.2 Serous borderline ovarian tumors (SBOT)**

SBOT are a rare disease. Population-based analysis of United States cases of SBOT diagnosed from 1992-1998 showed a low incidence of approximately 1.4 per 100,000 women per year (37). Moreover, analysis of age-specific incidence rates for SBOT showed that the incidence increases into the sixth decade of life and then stabilizes, possibly declining among women in the ninth decade (38). SBOT are neither clearly benign nor overtly malignant. Unlike benign tumors, SBOT display histologic features of malignancy, including nuclear atypia, cellular stratification and mitotic activity; however, the destructive stromal invasion observed in malignant tumors is not observed in SBOT (39-41). Histopathologically, although most SBOT are confined to the ovary when the disease has been diagnosed, in approximately one-third of women, non-invasive implants are present in the pelvis and/or abdomen. Clinically, SBOT exhibit a low proliferation rate; therefore, SBOT with non-invasive implants have been considered relatively indolent tumors with an excellent prognosis and a 5-year survival rate of up to 95% (41, 42). However, over a period of 15 years, recurrences of the disease does occur, and these relapses usually display the morphologic features of SBOT or LGSC and only rarely recur as HGSC (42-44).

Due to a past lack of culture systems and animal models, information about the biology of SBOT and their role in ovarian carcinogenesis has been largely lacking (38). In 1986, Crickard et al. maintained one SBOT on culture dishes coated with bovine corneal endothelial-secreted extracellular matrix (ECM) and demonstrated the protease activity of SBOT cells (45). In 1997, Luo et al. established long-term cultures of SBOT by infecting cells with adenovirus containing simian virus 40 (SV40) large T antigen (46). The cultured SBOT cells expressed *BRCA1* but not estrogen receptors and were not tumorigenic after subcutaneous injections in nude mice. Interestingly, cultured SBOT cells express matrix

metalloproteinases (MMP) and their inhibitors, tissue inhibitors of metalloproteinases (TIMP), but not urokinase-type plasminogen activator (uPA) or tissue-type plasminogen activator (tPA), which are found in the conditioned medium of invasive tumors (46). In 2005, Lee at al. developed a new ovarian cancer model by subrenal capsule xenografts of primary ovarian tumor tissues in immunodeficient mice. In that study, one SBOT tissue culture was established and maintained histopathological and immunohistochemical characteristics similar to the original tissues. Importantly, the margin between tumors and mouse renal tissues was clear, and it displayed the non-invasive properties of SBOT that resembled their behavior *in vivo* (47).

In our laboratory, four SBOT cell lines, designated SBOT1, SBOT2, SBOT3 and SBOT4, have been established from the tissues of four different patients. SBOT1 and SBOT2 cultures showed fibroblast contamination, whereas pure populations of epithelial cells were obtained from the SBOT3 and SBOT4 cultures. SBOT4 cells grew rapidly for 2-3 passages, but then became stationary and eventually died. Interestingly, a spontaneous permanent cell line was obtained in a subline of the SBOT3 culture, SBOT3.1 (48). Unlike normal OSE, which forms cobblestone monolayers of compact epithelial cells, cultured SBOT cells exhibit a whorled growth morphology with elongated and irregularly shaped cells that resemble cultured metaplastic OSE and LGSC (49, 50). Cultured permanent SBOT3.1 cells form colonies of flattened epithelial cells under sparse conditions, but when confluence increases, these cells become tightly packed and columnar (48). Intraperitoneal injection of SBOT3.1 cells in immunodeficient mice demonstrated that SBOT3.1 cells are not tumorigenic (48).

A *KRAS* or *BRAF* mutation has been detected in 68% of invasive LGSC and 61% of SBOT but not in HGSC, which characterizes SBOT and LGSC and indicates that LGSC and HGSC develop through independent pathways (51). Analysis of *KRAS* and *BRAF* genes

showed that SBOT3.1 cells contained no mutations in either *KRAS* or *BRAF*, whereas the

SBOT4 cells displayed the *V600E* mutation but were negative for *KRAS* mutations (48). Both

the SBOT3.1 and SBOT4 cells expressed the epithelial markers keratin and E-cadherin,

whereas only a small amount of N-cadherin was detected in the perinuclear regions (48).

Similar to the SBOT cultures established previously, SBOT3.1 and SBOT4 cells expressed

MMP2 and MMP9 (46, 48). Analysis of migratory and invasive abilities by scratch assay or

Matrigel-coated transwells showed that SBOT3.1 and SBOT4 cells have very limited

migratory and invasive capacities compared with two high-grade serous ovarian cancer cell

lines, SKOV3 and CaOV3 (48). These results indicate that SBOT cells retain their

non-invasive characteristics *in vitro*.


### 1.2.3 Progression from SBOT to invasive LGSC

Histopathological studies have proposed a dualistic model depicting the development of

serous ovarian carcinomas (Figure 1.1) (22, 23). In this model, invasive LGSC (also called

invasive micropapillary serous carcinoma, MPSC) develop in a stepwise manner from OSE

to benign serous cystadenoma, to SBOT and to non-invasive MPSC before becoming

invasive. In another pathway, HGSC develop from the OSE, inclusion cysts or distal portion

of the fallopian tube without morphologically recognizable intermediate stages (22, 23). The

indolent behavior of SBOT and LGSC distinguishes them from HGSC, which are aggressive

neoplasms and are the most common type of ovarian cancer. Compared with HGSC, LGSC

only accounts for a small proportion (9%) of all ovarian serous carcinomas (52).

It has been shown that approximately three-quarters of SBOT recur as invasive LGSC

that have a minimal response to therapy, and of those that recur, 47-74% of the patients will

die of the disease (42, 43). A clinicopathologic analysis of 135 cases of LGSC showed that

survival with stage I non-invasive and invasive LGSC was 100% and that survival for patients with stage II and III non-invasive and invasive LGSC with non-invasive implants was 80%. In contrast, the 5-year survival for women with invasive LGSC and invasive implants was 55% (53). These results indicate that a relatively poor prognosis was observed when non-invasive SBOT progressed to invasive LGSC.

**1.2.4 Low-grade serous versus high-grade serous ovarian carcinomas**

In the past decade, many studies using serial analysis of gene expression (SAGE) and gene expression microarrays have identified transcriptome-wide gene expression profiles for ovarian tumors and have shown that LGSC and HGSC are distinguishable based on their gene expression profiles (39, 54-58). Moreover, SBOT always clustered with LGSC rather than HGSC, which provides important evidence that SBOT and LGSC are closely related and that these tumors are distinct from HGSC. A number of genes that are involved in regulating cellular functions, including proliferation, apoptosis, migration and invasion, have been shown to be differentially expressed between HGSC and LGSC (21, 54).

Mutational analyses have shown that up to 80% of HGSC have *p53* mutations, whereas mutations in *p53* are very rare in SBOT and LGSC (59-62). These results suggest that a *p53* mutation is an early event in the development of HGSC. In contrast to HGSC, in which mutations in *KRAS* or *BRAF* are uncommon, more than half of SBOT and LGSC harbor activating mutations in *KRAS* or *BRAF* (33, 51, 63-65). These results demonstrate that mutations in *KRAS* and *BRAF* characterize both SBOT and LGSC. The Ras/Raf/MAPK signaling pathway plays an important role in the regulation of various cellular functions, and it has been shown that aberrant regulation of this pathway contributes to many human diseases, including cancer (66). MAPK is active in 71-81% of SBOT and LGSC, whereas

active MAPK was detected in only 41% of HGSC (67). *KRAS* mutations in codons 12 and 13 or a *BRAF* mutation in codon 600 (formerly identified as codon 599) have been identified in approximately two-thirds of SBOT and LGSC (51, 63, 64). The mutations in *KRAS* and *BRAF* are mutually exclusive (51). Interestingly, *KRAS* and *BRAF* mutations are not detected in isolated serous cystadenomas; however, they can be found in SBOT and in the adjacent cystadenoma epithelial cells in serous cystadenomas associated with small SBOT (68, 69). These results suggest that *KRAS* and *BRAF* mutations may be a key event that mediates the progression from serous cystadenomas to SBOT, and finally to LGSC. Taken together, these findings provide further evidence indicating that *KRAS* and *BRAF* mutations are largely restricted to SBOT and LGSC, suggesting that SBOT is a precursor of LGSC but not the more common HGSC.

## 1.3 Simian virus 40 (SV40) large T (LT) and small T (ST) antigens

### 1.3.1 Overview

Simian virus 40 (SV40) belongs to the *Polyomaviridae* family. Like the other members of *Polyomaviridae,* SV40 has a small icosahedral virion that contains a 5243 bp circular double-stranded DNA genome. In addition to the production of structural proteins, the Early Region of SV40 encodes two oncoproteins, large T antigen (LT) and small T (ST) antigen, which have been shown to play important roles in transformation (70). The SV40-induced transformation is mainly achieved by the inhibitory effects of LT on p53 and retinoblastoma protein (Rb) and the inhibitory effect of ST on protein phosphatase 2A (PP2A) (71, 72). It has been well documented that LT interacts with the DNA-binding domain of p53 and inhibits its transcriptional function by blocking the binding of p53 to its target promoters (73, 74). Rb is a tumor suppressor protein which can bind to the E2F transcription factor and

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inhibit its functions in cell cycle regulation and apoptosis (75). LT binds to Rb and blocks its ability to regulate E2F (76). ST binds to the scaffolding A subunit of PP2A and inhibits its phosphatase activity or changes the specificity of substrate by displacing the regulatory B subunit of PP2A (77, 78).

**1.3.2 p53 and Rb in the tumorigenesis of ovarian cancer**

Mutations in p53 and Rb are present in most types of human cancer (79). In HGSC, 50-80% of patients have *p53* mutations (21, 22, 24, 25). Although the *Rb* mutations in HGSC remain controversial, they are rare in SBOT and LGSC (80, 81). To date, several animal models have been established to examine the role of *p53* and *Rb* in the tumorigenesis of ovarian cancer (82).

The laying hen is a recognized model for ovarian cancer because of the possible development of spontaneous ovarian cancer. During 3.5 years of study, 32% (149/466) of hens developed ovarian tumors, and 8% (39/466) of the hens developed oviductal tumors (83). Histopathological analyses showed that four histological types (serous, endometrioid, mucinous and clear cell) of ovarian cancer were observed. However, in contrast to human ovarian cancer, in which serous ovarian cancer is the most common subtype, the serous and endometrioid subtypes were equally represented in the laying hen (84). Interestingly, ovarian cancers in laying hens exhibited *p53* mutations and *HER2* overexpression, thus resembling HGSC in humans (27). These findings indicate the important role of p53 in the tumorigenesis of ovarian cancer.

In mouse models, injections of *p53*-deficient OSE in combination with the overexpression of any two of the oncogenes, *c-myc*, *KRAS* or *Akt*, at subcutaneous, intraperitoneal, or ovarian sites induces ovarian cancer (85). A Cre-*loxP*-mediated conditional

knockout of *p53*, *Rb* or both by intrabursal administration of recombinant adenovirus expressing Cre showed that 33 of 34 (97%) mice with both genes knocked out developed ovarian tumors, whereas only 4 of 31 (13%) and 1 of 29 (3%) developed ovarian tumors after the single knockout of either *p53* or *Rb*, respectively (86). In another study, *p53* and *Rb* were inactivated in transgenic mice expressing the SV40 LT driven by the Mullerian inhibitory substance type 2 receptor promoter (*MISIIR*). In these transgenic mice, 18 of 36 (50%) female mice developed ovarian tumors. A growing number of authors have proposed that HGSC may arise from oviductal epithelial cells rather than from the OSE (87, 88). A recent study has shown that secretory epithelial cells in the human fallopian tube immortalized with human telomerase reverse transcriptase (hTERT) in combination with SV40 LT and ST antigens were transformed by either *HRAS^{V12}* or *c-myc* expression and gave rise to high-grade Müllerian carcinomas that were similar to human HGSC at the histological, immunophenotypical and genomic levels (89). Taken together, the findings from animal models strongly indicate that p53 and Rb play important roles in regulating the tumorigenesis of ovarian cancer.

**1.3.3 p53, Rb and PP2A in cell migration and invasion**

The roles of the tumor suppressor protein p53 in the regulation of the cell cycle, DNA repair and apoptosis have been well characterized (90). There is increasing evidence indicating that p53 can also regulate cell adhesion and migration (91-93). The effect of p53 on cell motility is mainly mediated by affecting the activity of Rho GTPases, including RhoA, Rac1 and Cdc42, which have been shown to play a key role in the regulation of actin cytoskeleton organization (94). In mouse embryonic fibroblasts (MEFs), RhoA, Rac1 and Cdc42 cooperated with p53 deficiency to enhance cell invasion (95, 96). In addition,

p53-deficient MEFs exhibited constitutive filopodia formation. In contrast, overexpression of wild-type p53 abolished the constitutively active Cdc42-induced cell migration and filopodia formation (94, 97).

Compared to p53, there are relatively few studies addressing the effect of Rb and PP2A on cell motility. One study showed that in breast cancer cells, the knockdown of Rb results in the down-regulation of E-cadherin and increases cell invasion. In addition, during EMT, the levels of Rb and p53 are significantly reduced, and the combined inactivation of Rb- and p53-activated Cdc42 leads to more actin cytoskeleton reorganization does than the inactivation of Rb alone (98). PP2A is a Ser/Thr phosphatase that regulates many cellular functions by modulating the activity of signal transduction pathways (99, 100). The inhibition of PP2A activity increased cell motility in human endothelial cells and mouse Lewis lung carcinoma cells by activating Src (101, 102). In MDCK cells, the expression of ST inhibited tight junction assembly and regulated Rho GTPases-mediated cytoskeleton reorganization, suggesting the involvement of PP2A in the regulation of cell motility (103). Furthermore, PP2A functions as a physiological calpain phosphatase to directly dephosphorylate μ- and m-calpains, which leads to decreased calpain activity and the suppression of the migration and invasion of human lung cancer cells (104).

## 1.4 Epithelial-mesenchymal transition (EMT)

### 1.4.1 Overview

The epithelial-mesenchymal transition (EMT) is a highly conserved biological process that controls morphogenesis in multicellular organisms (105). It was originally described as an epithelial-mesenchymal transformation in a model of chick primitive streak formation (106). The term transformation has now been replaced by transition to reflect the reversibility

of this process and to distinguish it from neoplastic transformation (107). The mesenchymal-epithelial transition (MET), the reverse process of EMT, is also involved in the regulation of morphogenesis (108). Currently, compared with EMT, relatively little is known about MET.

It is generally accepted that EMT can be classified into three different subtypes based on the biological context (105). Type I EMT is associated with implantation, embryo formation and organ development and neither causes fibrosis nor induces an invasive phenotype. This type of EMT can generate primary mesenchyme from primitive epithelium. Primary mesenchyme can then undergo MET to generate secondary epithelia. Type II EMT is related to wound healing, tissue regeneration and organ fibrosis. This type of EMT is strongly associated with inflammation. Type III EMT is involved in cancer progression and metastasis. Although these three types of EMT represent distinct biological processes, they have a common set of genetic and biochemical regulatory machinery. Generally, during EMT, a polarized epithelial cell undergoes multiple biochemical changes that allow it to acquire a mesenchymal cell phenotype, which includes the loss of polarity and cell-cell contacts, and increased migratory capacity, invasiveness and production of ECM (108).

**1.4.2 E- to N-cadherin switching in EMT**

The structure and polarity of epithelial cells require tight cell-cell and cell-matrix connections. Cadherins are the major component of epithelial adherens junctions that mediate cell-cell adhesion by calcium-dependent homophilic interactions of their extracellular domains (109, 110). The cadherin superfamily can be classified into at least six subfamilies based on protein domain composition, genomic structure and phylogenetic analysis of the protein sequences (111).

E-cadherin (epithelial cadherin, encoded by the *CDH1* gene) and N-cadherin (neuronal cadherin, encoded by the *CDH2* gene) belong to the classical (or Type I) cadherins (111). Cadherin switching involving the loss of the epithelial marker E-cadherin and the concomitant up-regulation of the mesenchymal marker N-cadherin has generally been considered the hallmark of EMT in cancer progression (112-114). In most epithelial cancers, the loss of E-cadherin is frequently found during tumor progression that is associated with metastasis and poor prognosis (115). Exogenous expression of N-cadherin has been shown to promote breast cancer cell migration and invasion (116-118). However, in contrast to the loss of E-cadherin, gain of N-cadherin is not always associated with EMT, which suggests that its role could be tumor-type dependent (115).

**1.4.3 Loss of E-cadherin**

The loss of E-cadherin is the most important event of EMT and is associated with epithelial tumor cell invasion (119). Down-regulation of E-cadherin can cause the mechanical disruption of adherens junctions. In addition to its physical role in maintaining epithelial cell structure and cell-cell adhesion, E-cadherin also acts as a negative regulator of the canonical Wnt signaling pathway, mainly via its interaction with β-catenin (120). E-cadherin-mediated cell-cell adhesion complexes are connected to the actin cytoskeleton via the E-cadherin cytoplasmic domain, β-catenin and α-catenin. Free cytosolic β-catenin is quickly turned over by interaction with a complex containing the adenomatous polyposis coli protein (APC), serine/threonine kinases CK1 and GSK3β, and the scaffold protein axin, which can phosphorylate β-catenin and then lead β-catenin to undergo ubiquitin-proteasome-mediated degradation (121). Activated Wnt signaling inhibits β-catenin degradation by phosphorylating and inhibiting GSK3β, which allows β-catenin to accumulate in the

cytoplasm and translocate into the nucleus (121). In the nucleus, β-catenin interacts with Tcf/Lef transcription factors and regulates the expression of a large number of genes involved in morphogenesis and cancer progression-related behaviors, such as cell proliferation, migration, and invasion (120).

As observed with many other proteins, E-cadherin can be regulated at the transcriptional and post-translational levels. To date, many molecular mechanisms have been identified that regulate E-cadherin expression and function, and most of these mechanisms act as negative regulators (119).

1.4.3.1 Transcriptional repression

Several transcription factors that strongly repress E-cadherin (*CDH1*) have been identified. They include the members of the snail family, Snail (SNAI1) and Slug (SNAI2); members of the basic helix-loop-helix (bHLH) family, Twist and the *E2A* gene products E12 and E47; and members of the zinc finger homeobox (ZFH) family, ZEB1 (δEF1) and ZEB2 (SIP1). These transcription factors bind to E-boxes located in the *CDH1* proximal promoter (122).

Snail zinc-finger transcription factors are involved in cell movement during embryonic development and in cell migration and invasion during cancer progression. The N-terminal domain of Snail family members is more divergent, whereas the C-terminal domain is highly conserved and is composed of four to six zinc fingers that mediate sequence-specific DNA binding to E-box elements (123). Deficiencies in Snail and Slug cause embryonic development failure in mouse and chick models (124, 125). Ectopic expression of Snail or Slug triggers EMT by repressing E-cadherin expression, whereas silencing of Snail expression reverses this process (126-130). It has been shown that both Snail and Slug can

bind E-box elements in the E-cadherin promoter; however, they recruit a different combination of co-repressors to repress the expression of E-cadherin (122).

The bHLH transcription factors bind to DNA as homo- or heterodimers using a consensus E-box site (122). The *E2A* gene encodes two bHLH transcription factors, E12 and E47, which are generated through differential splicing to either of two exons that encode the HLH domain (131). These transcription factors can bind the E-box elements in the E-cadherin proximal promoter. Ectopic expression of E12 or E47 down-regulates E-cadherin expression and enhances cell migration and invasion (127, 132). Twist proteins are highly conserved bHLH transcription factors that play important roles during embryogenesis. In mammals, Twist1 and Twist2 exhibit a high degree of sequence similarity (133). Ectopic expression of Twist1 or Twist2 down-regulates E-cadherin, up-regulates N-cadherin expression and promotes cell migration and invasion, suggesting that Twist proteins contribute to metastasis by inducing EMT (134, 135).

In vertebrates, the ZEB family contains two members, ZEB1 and ZEB2. ZEB1 is also known as δEF1 and is encoded by the *ZFHX1A* gene. ZEB2, also known as Smad-interacting protein 1 (SIP1), is encoded by the *ZFHX1B* gene (122). ZEB1 and ZEB2 both have two zinc-finger clusters at each end and a central homeodomain. The zinc-finger clusters mediate the binding of ZEB1 and ZEB2 to DNA that is composed of bipartite E-boxes. ZEB1 and ZEB2 can act as transcriptional activators or repressors by recruiting different transcriptional co-factors (136). During development, the expression of ZEB proteins has been detected in various tissues and, in most tissues, they can partially compensate for each other; however, ZEB1 can not compensate for ZEB2 in ZEB2-knockout-induced neural crest migration defects (137, 138). The ectopic expression of ZEB1 or ZEB2 down-regulates E-cadherin expression and promotes cell migration and invasion (139, 140). In epithelial cancer cells, the

induction of ZEB2 expression induces E- to N-cadherin switching and down-regulates the expression of tight junction, desmosome and gap junction proteins (141). These findings indicate that ZEB proteins act as crucial mediators of the EMT and contribute to cancer progression.

1.4.3.2 Post-translational regulation

E-cadherin mediated cell-cell adhesions are dynamically regulated. In addition to transcriptional repression, E-cadherin expression can be down-regulated by clathrin- or caveolin-mediated endocytosis (142-144). O-glycosylation of the cytoplasmic domain of newly synthesized E-cadherin blocks the transport of E-cadherin to the cell surface, resulting in reduced intercellular adhesion (145). Moreover, E-cadherin can be cleaved by a number of proteases, including MMPs, γ-secretase and caspase (146-149). p120 catenin interacts with the cleaved cytoplasmic domain of E-cadherin and enhances its translocation into the nucleus. In the nucleus, the cytoplasmic domain of E-cadherin modulates p120-Kaiso-mediated gene transcription and prevents staurosporine-induced apoptosis (150).

1.4.3.3 Genetic and epigenetic inactivation

Genomic mutations in the *CDH1* gene cause absent or truncated E-cadherin protein that is incapable of mediating cell-cell adhesion. Mutations in the *CDH1* gene have been identified in gastric, endometrial, ovarian and lobular breast cancer (151-153). In addition to genomic mutation, epigenetic regulation has also been reported to be involved in the regulation of E-cadherin expression. The 5' transcriptional start site of the *CDH1* gene promoter contains a dense CpG island (154). Hypermethylation of the CpG island in the *CDH1* gene promoter has been identified in various human cancers and is associated with

decreased E-cadherin expression and cancer metastasis (155-159).

### 1.4.4 Induction of EMT in cancer cells

During embryonic development and cancer progression, a large number of growth factors and agents can induce EMT in various cellular contexts (160). In cancer cells, EMT can be induced by both intrinsic and extrinsic signals (115). In intrinsic signaling, mutations in signal transduction molecules are sufficient to induce EMT and are necessary for triggering and maintaining the extrinsic stimulation-induced EMT. The introduction of a constitutively active mutant of *HRAS* is sufficient to induce and maintain EMT (161, 162). Mutations in the transforming growth factor-beta (TGF-β) receptor, Smad4 and MAPK pathways have been shown to modulate TGF-β-induced EMT (163-165).

In extrinsic signaling, the growth factors, cytokines and extracellular matrix proteins of the local microenvironment can induce cancer cells to undergo EMT (166, 167). To date, many growth factors and signaling pathways have been associated with EMT induction. These include TGF-β, epidermal growth factor (EGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), Wnt signaling and Notch signaling (166, 167). Upon stimulation, many signal transduction pathways can be activated to regulate EMT. Briefly, TGF-β activates the canonical Smad-dependent pathway or non-Smad pathways, which include ERK1/2, PI3K/Akt and Rho GTPases. EGF, HGF, IGF FGF and PDGF activate the Ras/Raf/MAPK, PI3K/Akt or Src/STAT pathways via receptor tyrosine kinases (RTKs). Jagged and Delta-like ligands activate the signal transcription factor CSL via Notch receptors. Wnt ligands bind to Frizzled receptors and inhibit GSK-3β, which leads to the formation of complexes of β-catenin with Tcf/Lef transcription factors. All of these signaling pathways

modulate gene expression and lead to EMT and cell migration and invasion (166, 167).

## 1.4.4.1 Epidermal growth factor (EGF)

The overexpression of the EGF receptor (EGFR) correlates with tumor aggressiveness and poor survival. EGF can induce cancer cell migration and invasion by activating downstream signaling such as the MAPK, PI3K/Akt, and FAK pathways (168). In addition, EGF is known to induce EMT in several types of human cancer cells by binding to its receptor, EGFR (169). Early effects of EGF signaling include the disruption of cell-cell adhesions and the induction of caveolae-dependent endocytosis of E-cadherin in tumor cells that overexpress EGFR. Chronic EGF treatment down-regulates caveolin-1, which leads to Snail-mediated down-regulation of E-cadherin transcription and enhanced cell invasion (144). In human breast cancer cells, EGF-induced EMT and cell invasion can be mediated by inducing Snail, ZEB1 and STAT3-dependent Twist (170-172). In the ovary, EGF can induce cell motility and EMT in OSE by activating ERK1/2 and the integrin-linked kinase (ILK) pathway (173). In ovarian cancer cells, EGF induces cancer cell motility and up-regulates the mesenchymal markers N-cadherin and vimentin by activating the JAK/STAT3 pathway (174).

## 1.4.4.2 Transforming growth factor-beta (TGF-β)

In the canonical TGF-β pathway, TGF-β binds to its type II receptor (TβRII) and recruits type I receptor (TβRI) to form a receptor complex. TβRII phosphorylates and activates TβRI. The activated TβRI subsequently phosphorylates receptor-regulated Smads, Smad2 and Smad3. Phosphorylated Smad2 and Smad3 then co-associate with Smad4, move into the nucleus and regulate gene expression by binding to Smad-specific elements in the

promoters of TGF-β-regulated genes (175).

The induction of EMT by TGF-β was first recognized in cultured mouse mammary gland epithelial cells (176). TGF-β treatment induces morphological changes from cuboidal to elongated spindle shapes. In addition, TGF-β decreases the expression of epithelial markers and concurrently increases the expression of mesenchymal markers (176). The TGF-β receptors and Smad proteins play important roles in TGF-β-induced EMT. In the induction of EMT, the activated Smad complex down-regulates epithelial marker gene expression and up-regulates mesenchymal gene expression by activating transcription factors of the Snail, bHLH and ZEB families (177). Inhibition of TβRI or TβRII functions by using dominant-negative forms of receptors or chemical inhibitors blocks TGF-β-induced EMT (178-182). Ectopic expression of Smad2 or Smad3 with Smad4 enhances TGF-β-induced EMT, whereas the ectopic expression of dominant-negative forms of Smad2, Smad3 or Smad4 blocks the induction of EMT by TGF-β (182).

In addition to the Smad-dependent pathway, TGF-β also can activate non-canonical, Smad-independent pathways. These include ERK1/2, p38 and JNK MAPK, Rho GTPases, and PI3K/Akt pathways, which have all been linked to TGF-β-induced EMT (177, 183). TGF-β treatment is able to directly activate MAPK signaling pathways, although the activation levels are generally much lower than those induced by RTKs. Many studies have shown that inhibition of ERK1/2, p38 and JNK MAPK activations blocks TGF-β-induced EMT (177). Rho GTPases, including Rho, Rac and Cdc42, are key molecules that regulate cytoskeleton organization, cell migration and gene regulation (184). TGF-β treatment increases the activated form of RhoA, which in turn activates the RhoA downstream effector Rho-associated protein kinase (ROCK). Inhibition of ROCK activity or the down-regulation of RhoA expression results in the inhibition of TGF-β-induced EMT and actin reorganization

(177). The activation of the PI3K/Akt axis has emerged as a central feature of the EMT (185). Inhibitors of PI3K/Akt signaling or the expression of a dominant-negative form of Akt are able to inhibit the TGF-β-induced morphological transition and the down-regulation of E-cadherin (177).

In cancer progression, TGF-β can function as a tumor suppressor during the early phase of tumorigenesis and can become a tumor promoter in the later phase (186). To date, few studies have shown that TGF-β treatment can induce EMT in human ovarian cancer cells. Treatment with TGF-β enhances cancer metastatic potential by inducing Smad3-dependent EMT (187). A recent study showed that TGF-β and EGF synergistically induce ovarian cancer cell motility and down-regulate E-cadherin by inducing the expression of Snail and Slug (188).

## 1.4.5 E-cadherin in ovarian cancer cell invasion

The loss of E-cadherin expression has been considered a fundamental event in EMT. This loss is consistently observed at sites of EMT during development and in cancer. Many studies have shown that the loss of E-cadherin increases the invasiveness of many types of cancer cells *in vitro* and contributes to the transition from adenoma to carcinoma in animal models. Clinically, the down-regulation of E-cadherin expression is often associated with tumor grade and stage (108, 160).

Mutation of E-cadherin is very rare in human ovarian cancer. Genetic screening revealed only one mutation in the E-cadherin gene out of 63 ovarian carcinomas (152). Interestingly, the expression of the transcriptional repressors of E-cadherin, Snail, Slug, Twist and ZEB1, has been shown to be up-regulated stepwise in benign, borderline, and malignant ovarian tumors. Therefore, these findings indicate that the loss of E-cadherin in

ovarian cancer is primarily regulated by transcriptional regulation.

In mouse ovarian tumor cells, high levels of E-cadherin are associated with low metastatic potential; in contrast, cells with high metastatic potential have relatively low levels of E-cadherin expression (189). Immunohistochemical studies have shown that E-cadherin is expressed in benign ovarian tumors but that the levels of E-cadherin are lower or undetectable in most borderline and malignant tumors. In addition, negative E-cadherin staining is associated with tumor grade, the presence of peritoneal seeding and a significantly poor patient survival (190, 191). Paired samples of ascites and solid ovarian tumors revealed a significant decrease in E-cadherin levels in the ascites cells compared with the respective solid tumors. The differences of E-cadherin levels also reflected the invasive capacity of the tumor cells *in vitro* (192). Several studies have demonstrated that reestablishing the expression of E-cadherin inhibits the invasiveness of epithelial cells (193, 194). In OVCAR3 ovarian cancer cells, overexpression of a dominant-negative E-cadherin disrupted adherens junctions and increased cell migration (195). Knockdown of E-cadherin by siRNA in ovarian cancer cells resulted in an up-regulation of α5-integrin mRNA and protein levels and permitted the cells to attach and invade more efficiently (196). In contrast, we have shown that the overexpression of E-cadherin inhibits basal invasiveness and diminishes EGF-induced invasion in ovarian cancer cells (197). Taken together, these findings support the notion that the loss of E-cadherin is involved in ovarian cancer cell invasion and associated cancer progression.

| Table 1.1 | |
|---|---|
| **Subtype** | **Relative frequencies** |
| **Major subtypes** | |
| Serous | 68-71% |
| Endometrioid | 9-11% |
| Clear cell | 11-12% |
| Mucinous | 3-6% |
| **Minor subtypes** | |
| Transitional | 1% |
| Undifferentiated | 5% |
| Mixed | 6% |

Adapted from McCluggage WG. *Pathology*. (2011) 43: 420-432.

**Table 1. 1 The histological subtypes of epithelial ovarian cancers**

| Table 1.2 | | |
|---|---|---|
| **Histology** | **Precursor Histology** | **Mutations** |
| **Type I** | | |
| **Low-grade serous** | **Serous borderline tunmor** | *KRAS, BRAF* |
| **Low-grade endometrioid** | **Endometriosis** | *CTNNB1, PTEN, PIK3CA* |
| **Clear cell** | **Endometriosis** | *PIK3CA* |
| **Mucinous** | **Mucinous borderline tumor** | *KRAS* |
| **Type II** | | |
| **High-grade serous** | **Inclusion cysts; Fallopian tube** | *p53, BRCA1/2* |
| **High-grade endometrioid** | **Not recognized** | *p53, BRCA1/2* |
| **Undifferentiated** | **Not recognized** | **Not recognized** |

Adapted from Cho KR. and Shih IeM. *Annu Rev Pathol.* (2009) 4: 287-313.

**Table 1. 2 Type I and Type II epithelial ovarian cancers**

**Figure 1. 1 Model of low-grade serous and high-grade serous carcinomas progression**

Development of ovarian low-grade serous (LGSC) and high-grade serous (HGSC) carcinomas involves two distinct pathways. Invasive LGSC develops in a stepwise manner, from benign serous cystadenoma through SBOT (serous borderline ovarian tumor), to non-invasive MPSC (micropapillary serous carcinoma) before becoming invasive. In another pathway, HGSC develop from the OSE, inclusion cysts or distal portion of the fallopian tube.

# Chapter 2. Rationale and objectives

Epithelial ovarian cancer is the most common form of ovarian cancer and the most lethal gynecological malignancy. Non-invasive serous borderline ovarian tumors (SBOT) can progress in a stepwise manner to invasive low-grade serous ovarian carcinomas (LGSC), whereas high-grade serous ovarian carcinomas (HGSC) can develop from the OSE or inclusion cysts (22). Recently, based on molecular and genetic studies, LGSC and HGSC have been considered to be two distinct entities (10). In the past few decades, researchers were mainly focused on the biology of HGSC. Relatively poor prognosis and survival were observed when non-invasive SBOT progressed to invasive LGSC. However, due to the current lack of appropriate culture systems or animal models of SBOT and LGSC, the molecular mechanisms that mediate the progression from non-invasive SBOT to invasive LGSC remain unknown.

In our laboratory, we established four SBOT cell lines from the tissues of four different patients (48). We attempted to immortalize the SBOT cells with SV40 LT/ST. Interestingly, after the introduction of SV40 LT/ST, the characteristic whorls and tightly packed columnar epithelial cells were replaced by a more atypical and scattered morphology. Immunostaining and western blot results showed that the cells lost the expression of E-cadherin and concurrently gained the expression of N-cadherin (198). These morphological and molecular changes indicated that SV40 LT/ST induced epithelial-mesenchymal transition (EMT) in cultured SBOT cells. Unexpectedly, we found that SV40 LT/ST significantly increased SBOT cell migration and invasion. These results indicated that SV40 LT and/or ST antigen can induce invasive and migratory behavior in SBOT cells, suggesting that SBOT cells have the potential to progress to invasive carcinomas and that signaling pathways regulated by SV40 LT and/or ST can contribute to this progression.

It is well known that SV40 LT inhibits p53 and Rb, whereas SV40 ST inhibits PP2A (71, 72). To distinguish the roles of p53, Rb and PP2A in the regulation SBOT cell invasion, p53, Rb and PP2A were inhibited individually. I infected the cell line SBOT4, which has a limited lifespan, with retroviruses encoding wild-type SV40 LT (inactivated p53 and Rb), ST (inactivated PP2A) or mutated forms of LT (inactivated p53 or Rb) (199). After retroviral infection and drug selection, none of the cultures infected with retroviruses encoding only ST or with mutated LT capable of inhibiting either p53 or Rb showed any difference compared with the control cultures in terms of morphology, growth rate or growth potential. The cell yield from these cultures was insufficient for the performance of invasion studies. However, one stable cell line that was infected with wild-type LT (SBOT4-LT) was established and proliferated for approximately 34 population doublings. These results suggested that the inhibition of both p53 and Rb is required for extending the lifespan of SBOT cells but is not sufficient to produce a permanent cell line. Moreover, the analysis of global gene expression profiles for SBOT, LGSC and HGSC suggested that progression from SBOT to LGSC may involve the attenuation of p53 signaling (54). Thus far, however, precise functional roles and associated molecular mechanisms of p53-mediated SBOT cell invasion remain unknown. Moreover, the effects of well-characterized EMT inducers, such as epidermal growth factor (EGF) and transforming growth factor-beta (TGF-β), on SBOT cell invasion remain to be defined.

## Aim of the study

**The general aim of my study was to examine the molecular mechanisms that control the progression from non-invasive SBOT to invasive LGSC.**


## The specific objectives of this study

**Objective 1: To investigate the role of p53 in SBOT cell invasion. (Presented in Chapter 3)**

1) To examine the effect of p53 inhibition on SBOT cell invasion

2) To determine the effect of p53 inhibition on PI3K/Akt signaling

3) To investigate the PI3K/Akt signaling-mediated transcriptional repression of E-cadherin

4) To examine the effect of E-cadherin knockdown on SBOT cell invasion

5) To examine the roles of p53, PI3K/Akt and E-cadherin in the cell invasion of the LGSC-derived cell line MPSC1.


**Objective 2: To investigate the regulatory role of p53 in E-cadherin promoter methylation. (Presented in Chapter 4)**

1) To examine E-cadherin promoter methylation in SBOT and MPSC1 cells

2) To investigate the effect of p53 inhibition on the expression of DNA methyltransferases (DNMTs) in SBOT cells

3) To examine the regulation of DNMT1 by p53-mediated PI3k/Akt signaling

4) To determine the contribution of DNMT1 to E-cadherin promoter methylation


**Objective 3: To investigate the role of EGF in SBOT and LGSC-derived MPSC1 cell invasion. (Presented in Chapter 5)**

1) To examine the expression of the EGF receptor in cultured SBOT and MPSC1 cells

2) To examine the effect of EGF on SBOT and MPSC1 cell migration and invasion

3) To investigate the downstream signaling pathways of EGF-induced cell migration and invasion

4) To determine the transcriptional repressors that mediate the EGF-induced down-regulation of E-cadherin


**Objective 4: To investigate the role of TGF-β in SBOT and LGSC-derived MPSC1 cell invasion. (Presented in Chapter 6)**

1) To examine the expression of TGF-β receptors in cultured SBOT and MPSC1 cells

2) To examine the effect of TGF-β on SBOT and MPSC1 cell and invasion

3) To investigate the downstream signaling pathways of TGF-β-induced cell invasion in SBOT cells and TGF-β-induced cell apoptosis in MPSC1 cells

4) To determine the transcriptional repressors that mediate the TGF-β-induced down-regulation of E-cadherin

# Chapter 3. Inhibition of p53 induces invasion of serous borderline ovarian tumor cells by accentuating PI3K/Akt-mediated suppression of E-cadherin

## 3.1 Introduction

Serous borderline ovarian tumors (SBOT) are the most common type of ovarian borderline tumors and are neither clearly benign nor obviously malignant. The recurrence of SBOT is often delayed, and when they do occur, the tumors typically show morphological features of SBOT or invasive low-grade serous carcinomas (LGSC), which are associated with a significantly poorer prognosis (200). Unlike benign tumors, SBOT show histological features of malignancy, including nuclear atypia, cellular stratification, mitotic activity and stromal microinvasion, but they lack the destructive stromal invasion seen in invasive high-grade serous carcinomas (HGSC) (44). An increasing number of studies support a model in which epithelial ovarian cancer progression is divided into two pathways. This model proposes that type I tumors tend to be invasive LGSC that arise from noninvasive SBOT, whereas type II tumors are invasive HGSC, which grow rapidly and without a definitive precursor lesion (33). Genetic studies have shown that *KRAS* and *BRAF* are frequently mutated in SBOT and LGSC but are rarely mutated in HGSC, whereas *p53* is usually normal in SBOT and LGSC but is frequently mutated in HGSC (60, 69).

Recently, a microarray study showed that SBOT do not exhibit any abnormalities in genes involving cellular proliferation, metastasis and chromosomal instability, which are associated with invasive HGSC. Rather, when compared with normal ovarian surface epithelium (OSE), SBOT are characterized by overexpressed p53 and changes in genes associated with the p53 pathway. In addition, analysis of the gene expression profiles of

invasive LGSC shows that they lack the increased p53 expression and signaling activity observed in SBOT (54). These observations suggest that the overexpression of p53 in SBOT plays an important role in the distinct phenotype associated with this lesion, and that the return of p53 to levels expressed in OSE is related to the progression of these noninvasive tumors to the more aggressive invasive LGSC.

We recently established an *in vitro* culture system with human SBOT cells. Cultured SBOT cells resembled LGSC in their levels of CA125 secretion, telomerase activation and E-cadherin expression. They secreted matrix metalloproteinases, but, in contrast to ovarian cancer cells, cultured SBOT cells grew slowly, were essentially non-invasive and exhibited limited motility, which are characteristics that resemble their behavior *in vivo*. Multicolor fluorescent *in situ* hybridization (mFISH) and microarray comparative genomic hybridization (CGH) analyses showed that the genes involved in p53 regulation and PI3K signaling were amplified (48). Most importantly, inactivation of p53, Rb and PP2A by the simian virus 40 (SV40) large (LT) and small (ST) T antigens allowed the cells to acquire characteristics associated with neoplastic progression, including increased cell motility and invasion and epithelial-mesenchymal transition (EMT) (198). Thus, transformation of SBOT cells to a more aggressive phenotype appears to be mediated in part by changes in the activity of p53, Rb and/or PP2A.

Phosphatidylinositol 3-kinase (PI3K) signaling has been shown to play a important role in several aspects of tumor progression, including cell migration, invasion and proliferation (201). Gain and/or amplification of the *PIK3CA* gene, which encodes the catalytic subunit of PI3K (p110α), and its increased expression are associated with enhanced PI3K activity in ovarian cancer cells (202). In addition, Akt, which is a major downstream target of PI3K, is amplified or activated in human tumors, including ovarian cancer (203). In many types of

human cancers, it has been shown that more than 80% of the mutations in *PIK3CA* gene are located at hotspots within two small conserved regions, the helical domain (encoded by exon 9) and the kinase domain (encoded by exon 20) (204). In ovarian cancer, a mutation analysis study shows that compared with endometrioid and clear cell ovarian carcinomas, serous carcinomas harbor less *PIK3CA* mutation, whereas, the *PIK3CA* is rarely mutated in the borderline tumor (205). Recently, it was shown that p53 directly binds to and transcriptionally inhibits a *PIK3CA* promoter (206). These results suggest that inactivation of p53 and subsequent up-regulation of *PIK3CA* contribute to the progression of ovarian cancer.

E-cadherin is a membrane glycoprotein located at cell adherens junctions. Loss of E-cadherin expression, which is a hallmark of EMT, is mainly due to the up-regulation of Snail, Slug, Twist, ZEB1 and other transcription factors, which repress E-cadherin (127, 128, 134, 166). Ovarian cancer cells with low E-cadherin expression are more invasive, and clinicopathological studies have shown that lack of E-cadherin is a predictor of poor clinical survival (190, 192). Recently, activation of the PI3K/Akt axis has emerged as a central feature of EMT. Squamous cell carcinoma cell lines exhibit constitutive activation of Akt, which down-regulates the expression of E-cadherin and promotes tumorigenicity and invasiveness (207). Such findings indicate that activation of the PI3K/Akt axis coupled with the down-regulation of E-cadherin expression may be important in promoting tumorigenesis.

In the present study, we first discriminated between the effects of p53 and Rb on the invasive ability of SBOT cells by individually inhibiting the corresponding genes. We found that inhibition of p53, Rb or both in combination allows SBOT cells to acquire a more aggressive phenotype. In addition, we show that inhibition of p53 alone is sufficient to increase the cell invasion of SBOT cells and to down-regulate the expression of E-cadherin, an effect which is mediated by increased p110α expression and Akt activation. Conversely,

enhancing the expression of p53 in invasive LGSC-derived MPSC1 cells decreased cell invasion and diminished p110α expression and Akt activation. These results suggest that p53 regulates the transition of SBOT cells from noninvasive to invasive ovarian carcinomas by activating the PI3K/Akt pathway and decreasing the expression of E-cadherin.

## 3.2 Material and methods

### Cell culture

Institutional approval for experimentation with human tissues, including informed consent from each subject, was obtained prior to initiating this study. SBOT cells were cultured in a 1:1 (v/v) mixture of M199/MCDB105 medium (Sigma-Aldrich, Oakville, ON) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories Inc., Logan, UT). The MPSC1 cell line, which was established from a LGSC (provided by Dr. Ie-Ming Shih, Department of Pathology, Johns Hopkins Medical Institutions, Baltimore, MD), was maintained in RPMI 1640 (Invitrogen, Burlington, ON) supplemented with 10% FBS (208).

### Retrovirus production and infection

Amphotropic retroviruses were produced by transfecting Phoenix retroviral packaging cells with either pBABE-neo-based vectors (Addgene, Cambridge, MA) encoding IRES-EGFP, the SV40 LT antigen, LT K1 (has an alteration in the Rb consensus binding motif) and LT delta 434-444 (has a deletion in part of the bipartite p53 binding domain) or with pBABE-zeo vectors encoding the SV40 ST antigen. All transfections were performed using Lipofectamine 2000 (Invitrogen). After transfection, the cells were purified by selection with 200 μg/mL G418 and 500 μg/mL zeomycin (199). The established SBOT4-LT cells were maintained in medium supplemented with 100 μg/mL G418.

**siRNA transfection and protein overexpression**

For the inactivation of p53, Rb, Slug and Twist, the cells were transfected with ON-TARGET*plus* SMART*pool* p53 (50 nM), Rb (50 nM), Slug (100 nM) and/or Twist (100 nM) siRNA (Dharmacon Research, Inc., Lafayette, CO) using Lipofectamine RNAiMAX (Invitrogen). The siCONTROL NON-TARGETING*pool* siRNA (Dharmacon) was used as the transfection control. For protein overexpression, a pIRES vector encoding the full-length wild-type mouse E-cadherin (provided by Dr. Carl Y. Sasaki, National Institute on Aging, Baltimore, MD), the pCMV-Neo-Bam-p53 vector (Addgene) and/or Myc-tagged DN-Akt (Upstate, Billerica, MA) were transfected into cells using Lipofectamine 2000.

**Immunofluorescence staining**

Cells were cultured on coverslips, fixed in methanol at -20 °C for 20 min, post-fixed in cold methanol/acetone (1:1) for 5 min and dried. Following rehydration in PBS, the coverslips were blocked with Dako Protein Block (Dako, Mississauga, ON) for 1 hr and incubated with antibodies to E-cadherin, p53, SV40 T antigen (Calbiochem, San Diego, CA) or keratin (rabbit anti-keratin, wide-spectrum screening; Dako) diluted in Dako Protein Block. Alexa 594-labeled goat anti-mouse or Alexa 488-labeled goat anti-rabbit IgG were used as a secondary antibody. Cells were counterstained with Hoechst 33258, rinsed with PBS, mounted with Gelvatol, and examined using a Zeiss Axiophot epifluorescent microscope equipped with a digital camera (Q Imaging, Burnaby, BC, Canada).

**Invasion assay**

The invasion assay was performed in Boyden chambers as described previously (209).

Filters were coated with 1 mg/mL growth-factor reduced Matrigel (BD Biosciences, Mississauga, ON). Cells in M199/MCDB105 medium supplemented with 0.1% FBS were incubated for 48 hr against a gradient of 10% FBS. Cells that penetrated the membrane were fixed with cold methanol, stained with Hoechst 33258, and the number of nuclei stained with Hoechst 33258 was counted using Northern Eclipse 6.0 software from Empix Imaging (Mississauga, ON, Canada). Each individual experiment had triplicate inserts and five microscopic fields were counted per insert.

**Western blots**

The cells were lysed, and the protein concentrations were determined using a protein assay kit with BSA standards according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA). Equal amounts of cell lysates were separated by SDS-PAGE and transferred to PVDF membranes. After blocking with TBS containing 5% non-fat dry milk, the membranes were incubated overnight at 4 °C with anti-p110 , anti-Rb, anti-phospho-Akt$^{Ser473}$, anti-Akt (all from Cell Signaling, Danvers, MA), anti-E-cadherin, anti-N-cadherin (both from BD Biosciences), anti-Twist, anti-p53 (both from Santa Cruz, Santa Cruz, CA), anti-Snail and anti-Slug (both from Abgent, San Diego, CA) antibodies. Afterwards, the membranes were incubated with HRP-conjugated secondary antibody. Immunoreactive bands were detected using the enhanced chemiluminescence (ECL) kit.

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

Total RNA was isolated using the RNeasy Plus kit (Qiagen, Mississauga, ON) according to the manufacturer's instructions. The RNA was reverse transcribed into first-strand cDNA (Amersham Pharmacia Biotech, Oakville, ON) following the manufacturer's instructions. The

primers used for SYBR Green reverse transcription-qPCR (RT-qPCR) were as follows: PIK3CA, 5'-TCA AAG GAT TGG GCA CTT TT-3' (sense) and 5'-GCC TCG ACT TGC CTA TTC AG-3' (antisense); E-cadherin, 5'-ACA GCC CCG CCT TAT GAT T-3' (sense) and 5'-TCG GAA CCG CTT CCT TCA-3' (antisense); N-cadherin, 5'-GGA CAG TTC CTG AGG GAT CA-3' (sense) and 5'-GGA TTG CCT TCC ATG TCT GT-3' (antisense); Snail, 5'-CCC CAA TCG GAA GCC TAA CT-3' (sense) and 5'-GCT GGA AGG TAA ACT CTG GAT TAG A-3' (antisense); Slug, 5'-TTC GGA CCC ACA CAT TAC CT-3' (sense) and 5'-GCA GTG AGG GCA AGA AAA AG-3' (antisense); Twist, 5'-GGA GTC CGC AGT CTT ACG AG-3' (sense) and 5'-TCT GGA GGA CCT GGT AGA GG-3' (antisense); p53, 5'-GTT CCG AGA GCT GAA TGA GG-3' (sense) and 5'-TCT GAG TCA GGC CCT TCT GT-3' (antisense); Rb, 5'-TCC CAT GGA TTC TGA ATG TG-3' (sense) and 5'-CCT TCT CGG TCC TTT GAT TG-3' (antisense) and GAPDH, 5'-GAG TCA ACG GAT TTG GTC GT-3' (sense) and 5'-GAC AAG CTT CCC GTT CTC AG-3' (antisense). RT-qPCR was performed on an Applied Biosystems 7300 Real-Time PCR System (Perkin-Elmer) equipped with a 96-well optical reaction plate. All RT-qPCR experiments were run in triplicate, and a mean value was used for the determination of mRNA levels. Relative quantification of the mRNA levels was performed using the comparative Ct method with GAPDH as the reference gene and with the formula $2^{-\Delta\Delta Ct}$.

**Statistical analysis**

The results are presented as the mean ± SEM of at least three independent experiments. The statistical analyses were conducted using a standard *t*-test for paired data. Multiple comparisons were first analyzed by one-way ANOVA, followed by Tukey's multiple comparison test. *P*-values of <0.05 were considered statistically significant.

## 3.3 Results

**Inhibition of p53 and Rb leads to morphological EMT and increases SBOT cell invasion**

As shown in Figure 3.1A, SBOT4 cells exhibited a whorled and irregular epithelial growth pattern, which is characteristic of this cell type. In contrast, SBOT4 cells infected with SV40 LT (SBOT4-LT) exhibited a more atypical and scattered morphology, indicating that EMT also occurs in the absence of ST. Immunostaining showed that all the SBOT4-LT cells were LT positive and continued to express keratin, confirming their epithelial origin (Figure 3.2). SBOT4 cells were essentially noninvasive, while SBOT4-LT cells were invasive at a level similar to the highly invasive ovarian cancer cell line SKOV3 (Figure 3.1B). SV40 LT inhibits p53 and Rb. To further discriminate between the effects of p53 and Rb on cell invasion, we knocked down endogenous p53, Rb or both genes. To make this experiment technically feasible, we used the spontaneously immortalized line, SBOT3.1, instead of SBOT4 cells because the latter have a limited life span. To confirm that this spontaneous immortalization did not affect the pathways examined in this study, the levels of target molecules were compared between SBOT3.1 and SBOT3.3 cells. These two cell lines are derived from the same patient, but SBOT3.3 is not spontaneously immortalized. Western blot analysis showed that the protein expression levels of the target molecules examined in this study did not differ significantly between SBOT3.1 and SBOT3.3 cells. In addition, their invasive ability was similar (Figure 3.3). Moreover, the RNA sequence results showed that *p53* is wild-type in SBOT3.1 cells (Data not shown).

The invasion assay showed that SBOT3.1 cells were noninvasive, whereas knockdown of p53 or Rb alone significantly increased cell invasion. However, knockdown of both p53 and Rb did not have any synergistic effects on cell invasion (Figure 3.1C). To further determine whether p53 is critical for the regulation of progression from noninvasive SBOT to

invasive low-grade serous carcinomas (LGSC), loss- and gain-of-function approaches were used to compare SBOT3.1 to MPSC1 cells, which were established from a LGSC. As shown in Figure 3.1D, MPSC1 cells were highly invasive, and overexpression of p53 significantly decreased their invasive ability. In addition, siRNA-mediated knockdown of p53 or Rb or overexpression of p53 did not significantly affect cell proliferation in SBOT3.1 and MPSC1 cells (Figures 3.4A and B). Moreover, overexpression of p53 did not increase apoptosis in MPSC1 cells (Figures 3.4C). These results suggest that overexpressed p53 represses cell invasion in SBOT and MPSC1 cells.

**Inhibition of p53, but not Rb, increases p110α expression and Akt activation**

To examine whether *PIK3CA* is wild-type or mutated in the SBOT cells, we sequenced the two hotspot-containing exons (9 and 20) in SBOT3.1 cells. The sequence results showed that no mutations were detected in exon 9 or 20 in SBOT3.1 cells, which confirmed that the *PIK3CA* mutation is rarely in borderline tumors (Data not shown). To determine whether the PI3K/Akt pathway contributes to the inhibition of p53-induced cell invasion, we analyzed the levels of *PIK3CA* in SBOT3.1 and MPSC1 cells. As shown in Figure 3.5A, knockdown of p53 increased the levels of *PIK3CA* in SBOT3.1 cells. Conversely, overexpression of p53 decreased the levels of *PIK3CA* in MPSC1 cells. Correlating with the measured mRNA levels, western blot analysis showed similar results for both SBOT3.1 and MPSC1 cells (Figure 3.5B). In contrast, knockdown of Rb in SBOT3.1 cells did not affect the level of *PIK3CA* (Figure 3.5C). In addition, the levels of *PIK3CA* and p110α were increased in SBOT4-LT cells compared with SBOT4 cells (Figure 3.6).

Because Akt is the major downstream target of PI3K, we examined the influence of p53 on the activation of Akt. siRNA-mediated knockdown of p53 in SBOT3.1 cells led to

increased Akt phosphorylation in response to FBS. Conversely, overexpression of p53 diminished FBS-induced Akt phosphorylation in MPSC1 cells (Figure 3.7A). To further confirm the effects of PI3K/Akt on cell invasion, SBOT3.1 cells were treated with the PI3K inhibitor LY294002. As shown in Figure 3.7B, LY294002 inhibited basal levels of cell invasion. Interestingly, LY294002 also inhibited p53 knockdown-induced cell invasion. The involvement of PI3K in the inhibition of p53-induced cell invasion was confirmed using another PI3K inhibitor, wortmannin (Figures 3,7C). In SBOT4-LT cells, transfection of SBOT4-LT cells with dominant-negative Akt (DN-Akt) significantly decreased cell invasion (Figure 3.8A). In addition, LY294002 and wortmannin inhibited cell invasion in a dose-dependent manner (Figures 3.8B and C). These results indicate that inhibition of p53 increases cell invasion by up-regulating the PI3K/Akt pathway. Interestingly, inhibition of Rb also enhanced invasiveness, but did not affect the levels of *PIK3CA* suggesting that it may act by a different pathway.

**Inhibition of p53 down-regulates E-cadherin**

To characterize the role of p53 in the regulation of EMT, we analyzed the levels of E-cadherin in SBOT3.1 and MPSC1 cells. As shown in Figures 3.9A and B, siRNA-mediated knockdown of p53 decreased the levels of E-cadherin mRNA and protein in SBOT3.1 cells. Conversely, overexpression of p53 increased the levels of E-cadherin mRNA and protein in MPSC1 cells. Immunostaining showed that manipulation of p53 did not significantly change the localization of E-cadherin in either SBOT3.1 or MPSC1 cells (Figure 3.9C). In contrast, siRNA-mediated knockdown of p53 increased the expression of the mesenchymal marker N-cadherin in SBOT3.1 cells and N-cadherin was decreased in p53-overexpressing MPSC1 cells (Figure 3.10). However, siRNA-mediated knockdown of

Rb did not affect the mRNA level of E-cadherin (Figure 3.11). To elucidate the mechanism responsible for the transcriptional regulation of E-cadherin, we examined the expression of Snail, Slug, Twist and ZEB1 which repress E-cadherin transcription. Knockdown of p53 increased the mRNA levels of Slug and Twist in SBOT3.1 cells. Conversely, overexpression of p53 decreased the mRNA levels of Slug and Twist in MPSC1 cells (Figures 3.9D). Correlating with the measured mRNA levels, western blot analysis showed similar results for both SBOT3.1 and MPSC1 cells (Figure 3.9E). However, the manipulation of p53 expression did not change the levels of Snail and ZEB1 in either SBOT3.1 or MPSC1 cells (Figures 3.9D and E). To confirm whether the p53 knockdown-mediated down-regulation of E-cadherin was mediated by Slug and Twist, we examined the levels of E-cadherin protein in SBOT3.1 cells transfected with p53 siRNA in combination with Slug or Twist siRNA. The results showed that siRNA-mediated knockdown of Slug or Twist abolishes the down-regulation of E-cadherin induced by knockdown of p53. These results confirmed that Slug and/or Twist regulate p53-mediated expression of E-cadherin in SBOT cells (Figure 3.9F). Consistent with the results obtained in SBOT3.1 cells, the levels of E-cadherin mRNA and protein decreased while those of Slug and Twist increased in SBOT4-LT cells compared with SBOT4 cells. In addition, the levels of N-cadherin mRNA and protein increased in SBOT4-LT cells compared with SBOT4 cells. Similarly, the levels of Snail remained unaltered in SBOT4 and SBOT4-LT cells (Supplemental Figure 3.12).

**Activation of the PI3K/Akt pathway is involved in the inhibition of p53-down-regulated E-cadherin**

SBOT3.1 cells were treated with p53 siRNA and LY294002. As shown in Figure 3.13A, down-regulation of E-cadherin caused by siRNA-mediated knockdown of p53 was abolished

by treatment of the cells with LY294002. Additionally, LY294002 diminished the increase of Slug and Twist caused by siRNA-mediated knockdown of p53. To further confirm the role of Akt in down-regulating E-cadherin, MPSC1 cells were transfected with DN-Akt. Western blot analysis showed that DN-Akt decreased the levels of E-cadherin and increased the levels of Slug and Twist in MPSC1 cells (Figure 3.13B). Furthermore, invasion assays showed that DN-Akt significantly decreased cell invasion of MPSC1cells (Figure 3.13C).

**E-cadherin represses invasion in SBOT and MPSC1 cells**

To further examine the role of E-cadherin in the progression from noninvasive SBOT to invasive LGSC, we knocked down endogenous E-cadherin in SBOT3.1 cells. siRNA-mediated knockdown of E-cadherin resulted in a decrease in E-cadherin protein levels and increased the cell invasion of SBOT3.1 cells (Figure 3.14A). To further test our hypothesis that E-cadherin represses cell invasion, we decided to reverse the loss of E-cadherin by overexpressing E-cadherin. However, as we observed significant effects of p53 on endogenous E-cadherin, we overexpressed E-cadherin by using an E-cadherin construct lacking regulatory elements. As shown in Figure 3.14B, overexpression of E-cadherin abolished the increased cell invasion of SBOT3.1 cells induced by knockdown of p53. In MPSC1 cells, overexpression of E-cadherin decreased cell invasion (Figure 3.14C). In SBOT4-LT cells, overexpression of E-cadherin showed the similar results in cell invasion (Figure 3.15). These results indicated that E-cadherin plays an important role in the p53-regulated cell invasion in SBOT and MPSC1 cells.

## 3.4 Discussion

Noninvasive SBOT and invasive LGSC are thought to represent different stages in a tumorigenic continuum and to develop along pathways distinct from invasive HGSC (54). In

the present study, we attempted to discriminate between the effects of p53 and Rb on the neoplastic progression of SBOT cells by selectively inhibiting the corresponding genes. We demonstrated that inhibition of p53 or Rb alone significantly increased the invasion of SBOT cells. Inhibition of p53-induced cell invasion was mediated by activation of the PI3K/Akt pathway and by the down-regulation of E-cadherin. In contrast, inhibition of Rb did not affect the level of *PIK3CA* and E-cadherin. These results not only indicate that the progression from SBOT to invasive LGSC involves the attenuation of p53 and Rb signaling but also point to an unexpected function of p53 in SBOT cells, which is to suppress EMT and the transition to invasive ovarian carcinomas.

Mutation of p53 is the most common genetic alteration in invasive HGSC, but it is rare in borderline tumors and invasive LGSC (60, 210). Our sequence results confirmed that SBOT cells harbor wild-type p53. Recently, a microarray study showed that compared with normal OSE, SBOT are characterized by increased p53 expression levels. In addition, the level of p53 reverts back to normal when SBOT progress to invasive LGSC suggesting that the return of p53 to levels expressed in OSE may be related to the progression of these noninvasive tumors to more aggressive invasive LGSC (54). In the present study, we showed that SV40 LT- or p53 siRNA-mediated inhibition of p53 activity increases the cell invasion of SBOT cells, while increasing the activity of p53 in invasive LGSC had the opposite effect. These results indicate that progression from noninvasive SBOT to invasive LGSC involves the attenuation of p53 signaling.

Although p53 has been extensively studied for its role in the control of cell cycle checkpoints and apoptosis, recent studies suggest that it is responsible for other cellular functions, including cell adhesion, inhibition of angiogenesis, cell migration, cell fate and cytoskeleton organization (93). We previously showed that SBOT cells express MMP2,

MMP9 and variable levels of active uPA and that the SV40 LT/ST-induced invasion in SBOT cells is not associated with changes in protease secretion (198). Thus, inhibiting p53 appears to increase cell motility but does not modulate protease activity.

Loss of Rb function is also a crucial event in tumorigenesis. A recent study showed that in breast cancer cells depletion of Rb downregulates E-cadherin expression and induces Cdc42-mediated actin cytoskeleton reorganization and cell invasion. In addition, during EMT, Rb and p53 expression levels are both significantly down-regulated. Their results suggest that loss of Rb down-regulates the expression level of E-cadherin, whereas in cells with disrupted cell-cell interactions, loss of p53 can increase cell survival and may also facilitate cell motility (98). In present study, knockdown of Rb alone increased the invasion of SBOT3.1 cells. However, inhibition of Rb did not affect the levels of *PIK3CA* and E-cadherin. This would suggest the presence of additional, E-cadherin-independent, mechanisms for inhibition of Rb-induced cell invasion in SBOT cells.

The PI3K/Akt pathway is important in ovarian carcinogenesis and is implicated in the regulation of cell migration, invasion and EMT (185, 211). Interestingly, increases in PI3K mRNA and protein levels are more frequent than are increases in gene copy number, suggesting that copy number-independent mechanisms also regulate the levels of PI3K expression in ovarian cancer cells. Here, our sequence results show that no *PIK3CA* mutations were detected in SBOT cells. In addition, a recent study showed that p110α expression and Akt activation are negatively controlled by p53 (206). In the present study, we demonstrate that p53 also suppresses p110α expression and Akt activation in SBOT cells and that inhibition of p53-induced SBOT cell invasion was abolished when the cells were treated with the PI3K inhibitors LY294002 and wortmannin or when transfected with DN-Akt. Taken together, these results indicate that p53-mediated suppression of PI3K/Akt

signaling may be a critical factor that regulates the progression of SBOT to the invasive phenotype.

The defining characteristic of EMT is a down-regulation in E-cadherin levels and concurrent up-regulation of N-cadherin (212). In the present study, inhibition of p53 induced a switch from E-cadherin to N-cadherin expression. However, our previous study showed that overexpression of N-cadherin does not induce cell invasion in SBOT cells (198). Several studies have demonstrated that re-establishing the expression of E-cadherin in epithelial tumor cells results in a reversion from an invasive to a noninvasive phenotype (193, 194). It has been shown that loss of E-cadherin and p53 accelerates the development of metastatic mammary carcinomas in mice (213). In the present study, we showed that knockdown of E-cadherin induced invasiveness of SBOT3.1 cells while overexpression of E-cadherin decreased MPSC1 cell invasion. Moreover, SBOT cell invasion, induced by inhibition of p53, was abolished by overexpression E-cadherin. Thus, our results demonstrate, for the first time, that E-cadherin suppresses the invasion of SBOT cells and that it plays a role in the progression from noninvasive SBOT cells to invasive tumors.

Recently, a study showed loss of transcriptional activity of p53 down-regulates E-cadherin expression by up-regulation of Slug and ZEB1 in colon carcinoma cells. Their results suggest that inactivation of p53 contributes not only to cellular growth but permits tumor progression towards an invasive phenotype (214). In the present study, we demonstrated that p53 increases E-cadherin expression by repressing the expression of Slug and Twist, but not Snail and ZEB1. It has been shown that wild-type p53 can suppress cancer cell invasion by inducing MDM2-mediated Slug degradation, but p53 does not influence the expression of Slug mRNA. In addition, mutations in *p53* correlate with low expression of MDM2, high expression of Slug and low E-cadherin levels in non-small cell lung cancer

(215). Taken together, these results suggest that p53 may regulate Slug and Twist at the transcriptional and post-translational levels. However, the detailed mechanism of this regulation requires further investigation.

In summary, our study demonstrates that inhibition of p53 induces cell invasion by up-regulating the PI3K/Akt pathway and down-regulating the expression of E-cadherin in SBOT cells. These results suggest that p53, which is characteristically overexpressed in SBOT (6), acts as an ovarian epithelial tumor suppressor by inhibiting the progression of SBOT to invasive ovarian carcinomas.

**A**



**B**



**C**
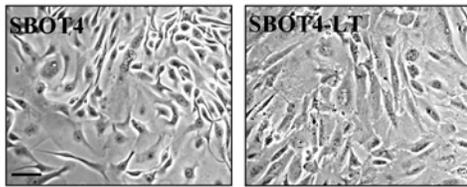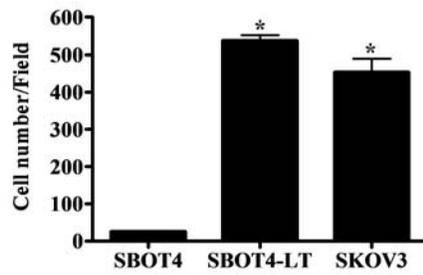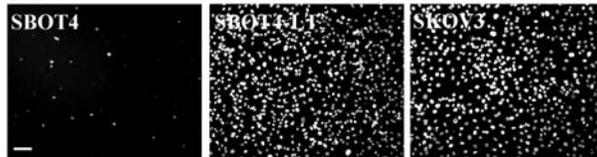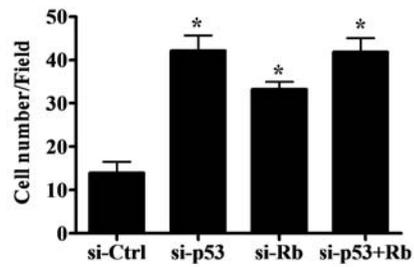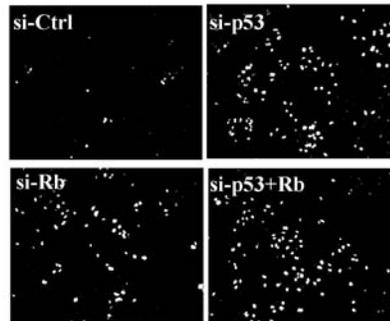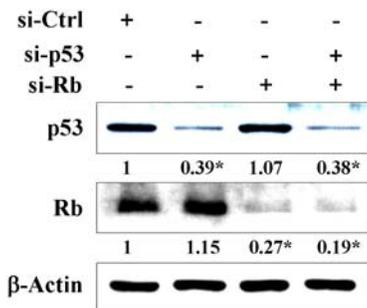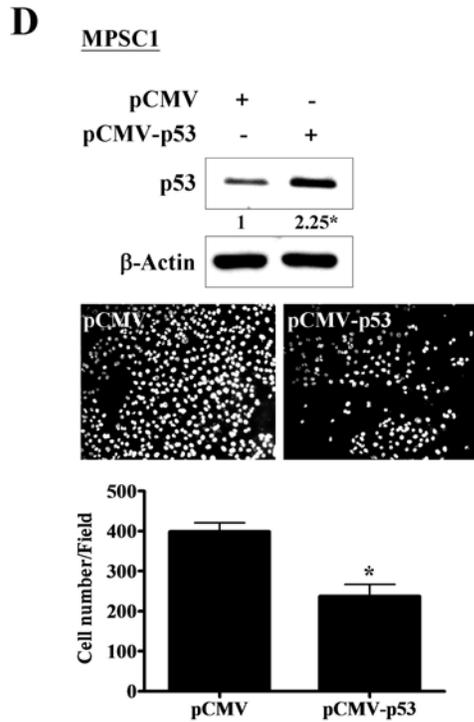
**Figure 3. 1 Inhibition of p53 and/or Rb changes the morphology and increases the cell invasion of SBOT cells**

(A) The morphology of SBOT4 and SBOT4-LT cells. (B) Matrigel-coated transwell assay. The upper panel shows representative fluorescence images of the invasion assay; the lower panel shows a summary of the statistical analysis. (C) SBOT3.1 cells were transfected for 48 hr with siRNA against p53, Rb or both. The non-targeting siRNA (si-Ctrl) was used as the control. The levels of the p53 and Rb proteins were analyzed by western blot. The effects of siRNA-mediated knockdown of p53 and Rb on the cell invasion of SBOT3.1 cells were analyzed by culturing the cells in Matrigel-coated transwells. (D) MPSC1 cells were transfected for 48 hr with control (pCMV) or p53-encoding (pCMV-p53) vectors, and the levels of p53 protein were analyzed by western blot. The cell invasion of p53-overexpressing MPSC1 cells was analyzed by culturing the cells in Matrigel-coated transwells. Numbers under the western blots represent the densitometry quantifications. The results are expressed as the mean ± SEM of at least three independent experiments. *p<0.05 compared with SBOT4, si-Ctrl or pCMV. The scale bar represents 100 μm.

| Keratin | SV40-LT | Hoechst | Merge |
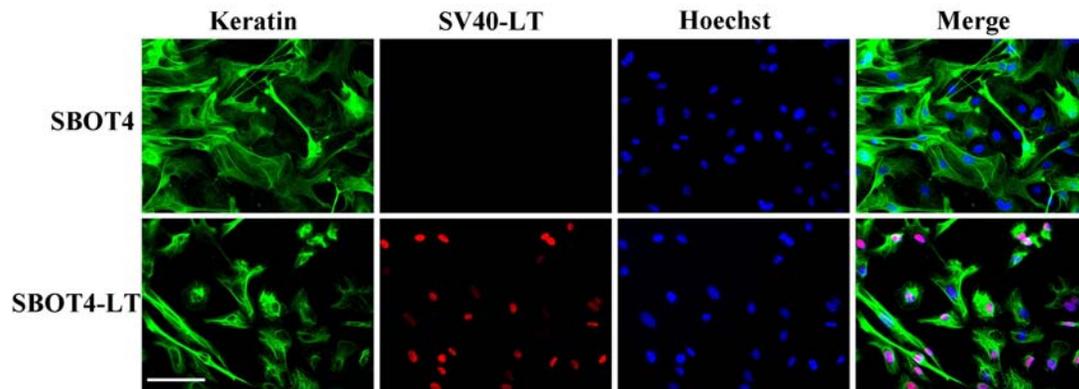
**Figure 3. 2 Expression of SV40 in SBOT4-LT cells**

Cells were seeded on the coverslips and expression of keratin and SV40 LT was examined by immunofluorescence staining. Nuclei were stained with Hoechst 33258. Scale bar represents 100 μm.
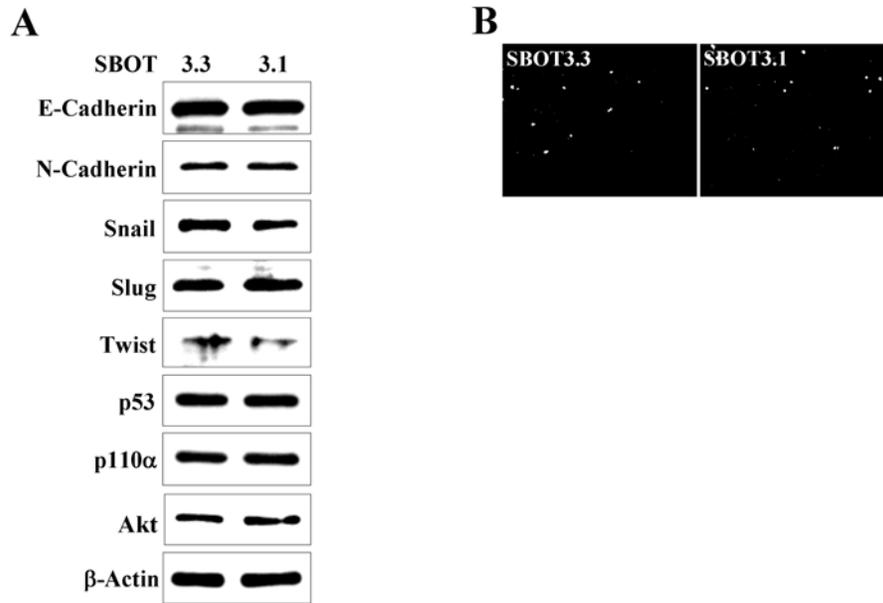
**Figure 3. 3 Expression of targeted proteins and cell invasiveness in SBOT3.1 and SBOT3.3 cells**

**(A)** The protein levels of target molecules were examined by western bolt. **(B)** Matrigel-coated transwell assay.
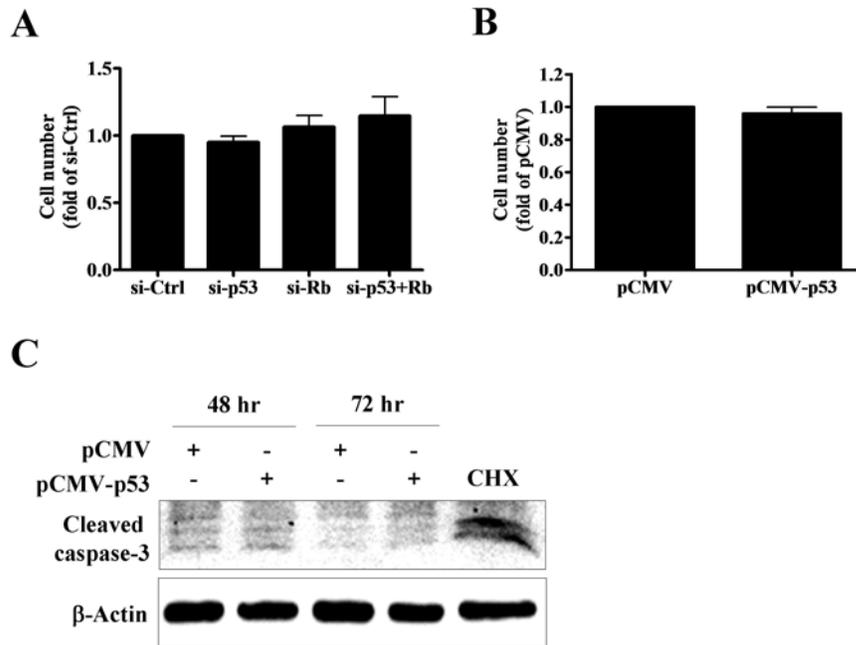
**Figure 3. 4 Knockdown and overexpression of p53 does not affect cell proliferation and apoptosis**

**(A)** SBOT3.1 cells were transfected with p53, Rb or both siRNAs for 72 hr and the numbers of cells were counted by trypan blue. **(B)** MPSC1 cells were transfected with pCMV-p53 for 72 hr to overexpress p53 and the numbers of cells were counted by trypan blue. **(C)** MPSC1 cells were transfected with pCMV-p53 for 48 and 72 hr to overexpress p53 and apoptosis was determined by western blot using cleaved-caspase-3 antibody. Cells treated with cycloheximide (100 µM) according to the manufacturer's procedure of Ready-to-use Apoptosis Inducer kit (BioVision, Mountain View, CA) was used as positive controls. Results are expressed as mean ± SEM of three independent experiments.
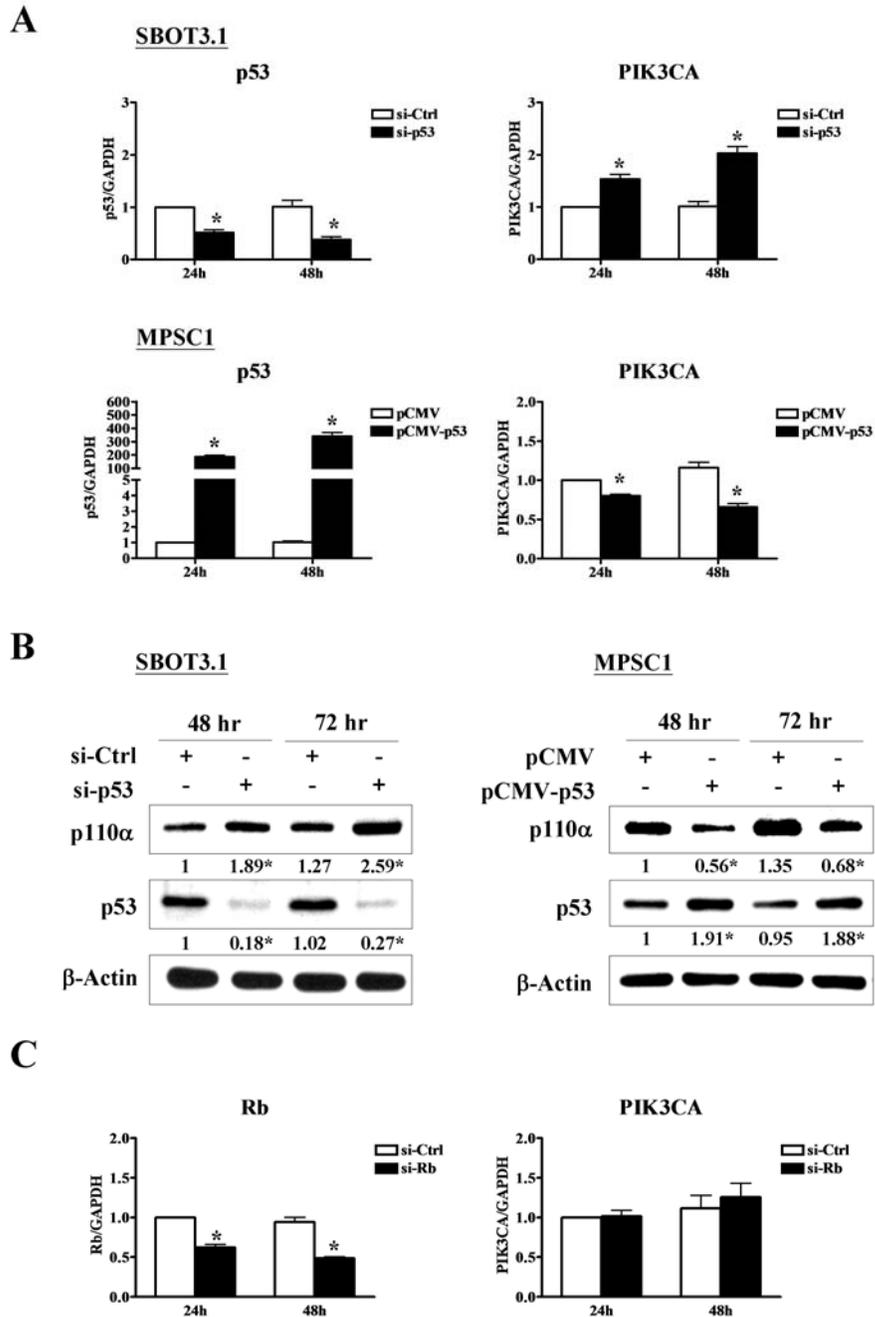
**Figure 3. 5 Inhibition of p53, but not Rb, increases the expression of p110α**

**(A and B)** The levels of *PIK3CA* and p110α were analyzed by RT-qPCR and western blot in p53-knocked down SBOT3.1 cells and p53-overexpressed MPSC1 cells. **(C)** SBOT3.1 cells were transfected with Rb siRNA. The levels of Rb and *PIK3CA* were analyzed by RT-qPCR. Numbers under the western blots represent the densitometry quantifications. The results of the RT-qPCR are expressed as the mean ± SEM of three independent experiments. *p<0.05 compared with si-Ctrl or pCMV.
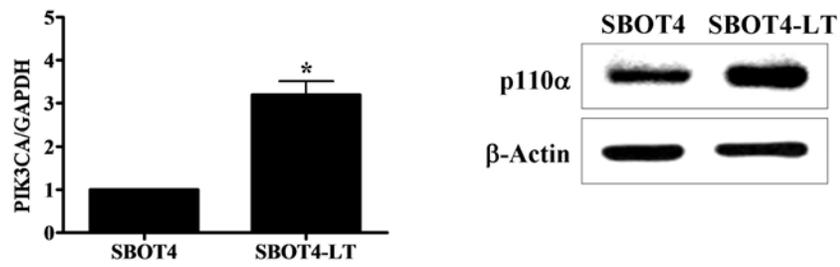
**Figure 3. 6 SV40 LT increases *PIK3CA* ana p110α in SBOT4 cells**

The levels of *PIK3CA* and p110α in SBOT4 and SBOT4-LT cells were analyzed by
RT-qPCR (left panel) and western blot (right panel).
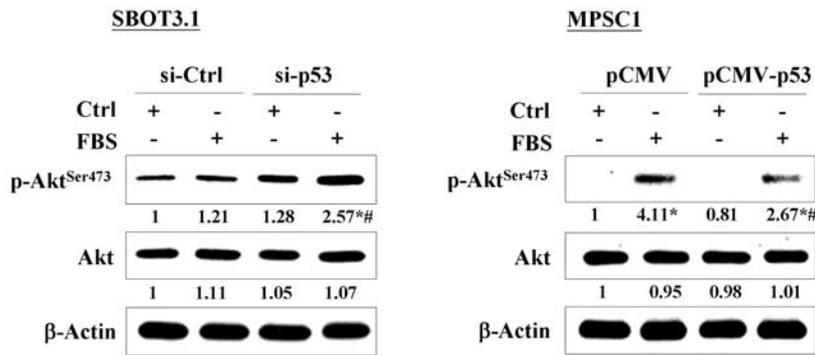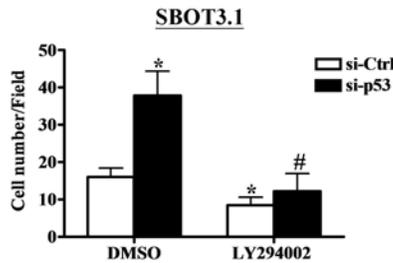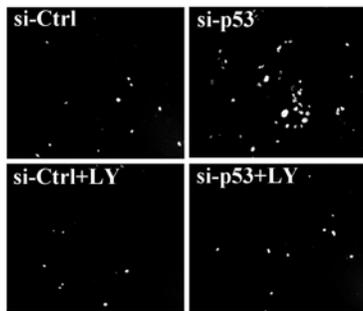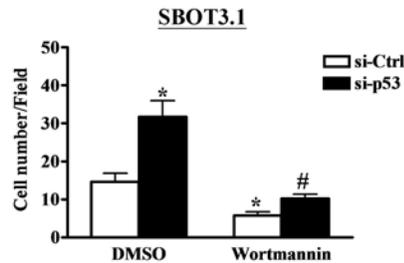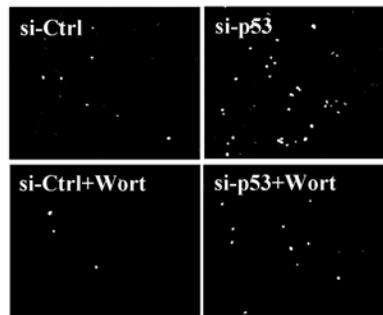
**Figure 3. 7 Activation of the PI3K/Akt pathway is required for the inhibition of p53-induced cell invasion**

**(A)** p53-knocked down SBOT3.1 cells and p53-overexpressing MPSC1 cells were subjected to serum starvation overnight, after which they were treated with medium containing 10% FBS for 10 min. Akt activation was analyzed by western blot. **(B)** SBOT3.1 cells transfected with control or p53 siRNA were treated with 10 μM LY294002 for 30 min and then the cell invasion was analyzed by Matrigel-coated transwells. **(C)** SBOT3.1 cells transfected with control or p53 siRNA were treated with 5 μM wortmannin for 30 min and then the cell invasion was analyzed by Matrigel-coated transwells. Numbers under the western blots represent the densitometry quantifications. The results are expressed as the mean ± SEM of at least three independent experiments. *$p<0.05$ compared with DMSO-treated si-Ctrl. #$p<0.05$ compared with DMSO treated si-p53.
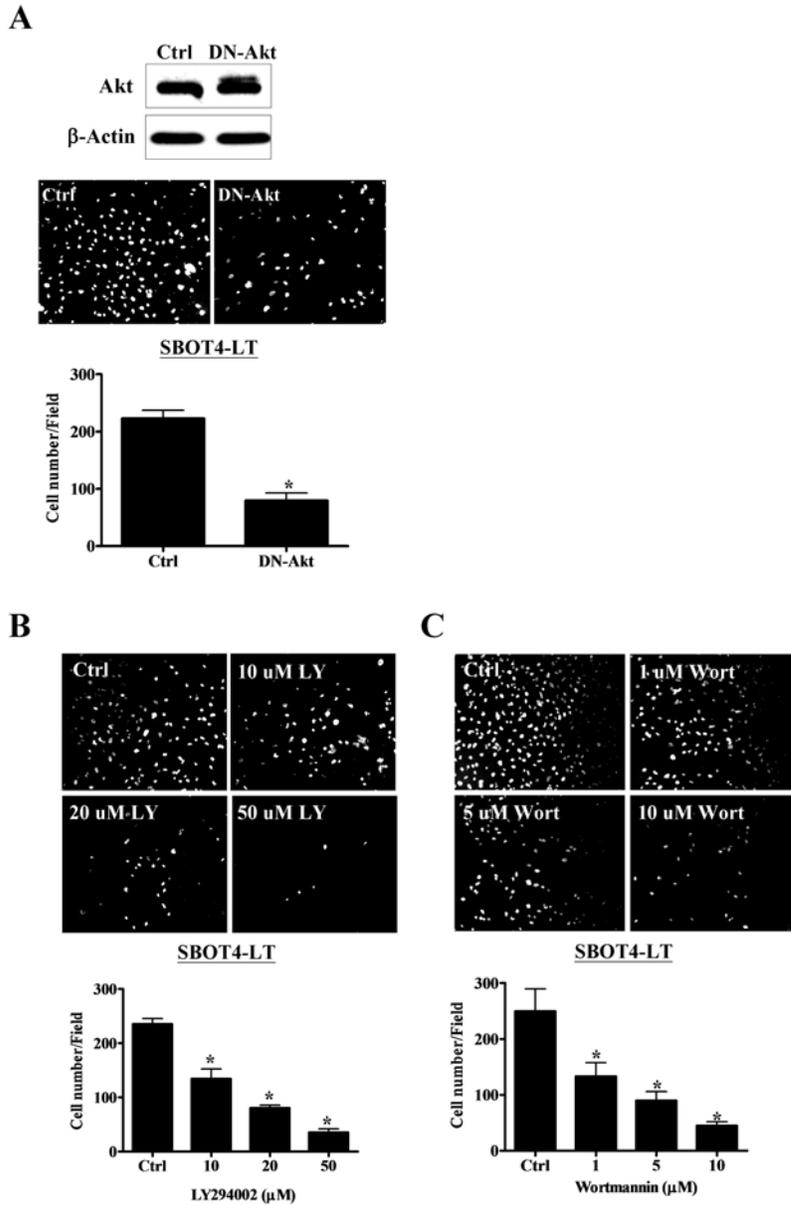
**Figure 3. 8 Activation of the PI3K/Akt pathway is required for the inhibition of SV40 LT-induced cell invasion**

**(A)** SBOT4-LT cells were transfected with DN-Akt for 48 hr and then the cell invasion was analyzed by Matrigel-coated transwells. The expression level of Myc-tagged DN-Akt was analyzed by western blot using Akt antibody. **(B)** SBOT4-LT cells were treated with different concentrations of LY294002 for 30 min and then the cell invasions were analyzed by Matrigel-coated transwells. **(C)** SBOT4-LT cells were treated with different concentrations of wortmannin for 30 min and then the cell invasions were analyzed by Matrigel-coated transwells.

**A**

SBOT3.1

E-Cadherin

☐ si-Ctrl
■ si-p53

MPSC1

E-Cadherin

☐ pCMV
■ pCMV-p53

**B**

SBOT3.1

| si-Ctrl | + | − |
| si-p53 | − | + |

E-Cadherin
1    0.60*

p53
1    0.25*

β-Actin

MPSC1

| pCMV | + | − |
| pCMV-p53 | − | + |

E-Cadherin
1    1.65*

p53
1    2.12*

β-Actin

**C**

SBOT3.1

si-Ctrl | si-p53

MPSC1

pCMV | pCMV-p53

E-cadherin/p53/Hoechst

55

**D**

<u>SBOT3.1</u>



<u>MPSC1</u>



**E**

**F**

| si-Ctrl | + | - | - | - | - | - |
| si-p53 | - | + | - | - | + | + |
| si-Slug | - | - | + | - | + | - |
| si-Twist | - | - | - | + | - | + |

E-Cadherin

1　　0.55*　0.91　0.90　0.83#　0.80#

p53

1　　0.14*　1.12　0.97　0.20*　0.11*

Slug　　←

1　　1.67*　0.12*　1.05　0.09*　0.94

Twist

1　　1.54*　1.02　0.06*　0.91　0.02*

β-Actin
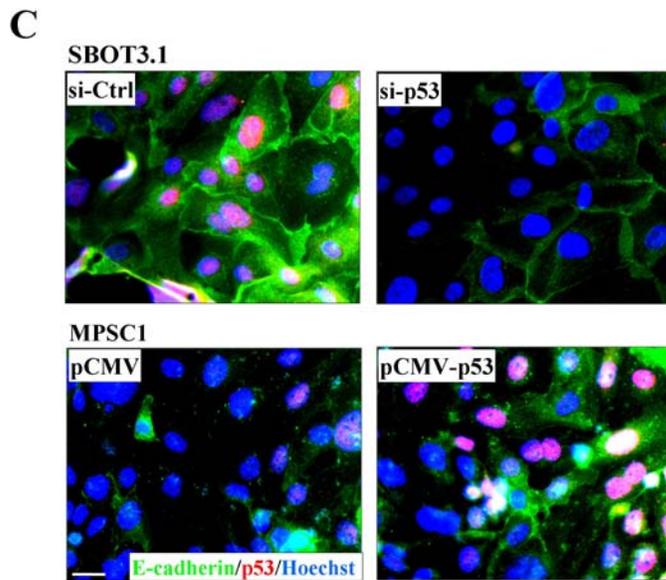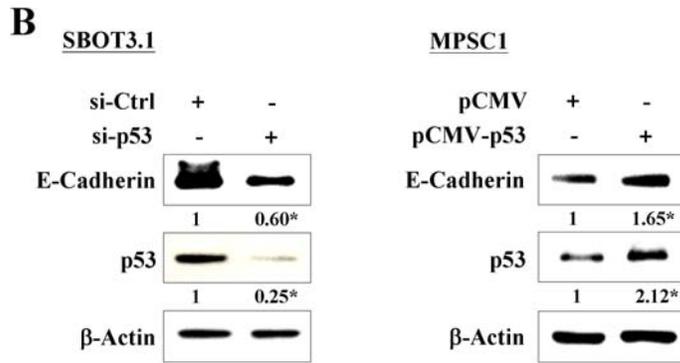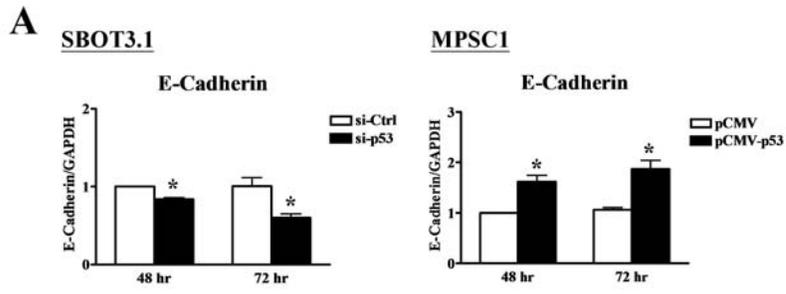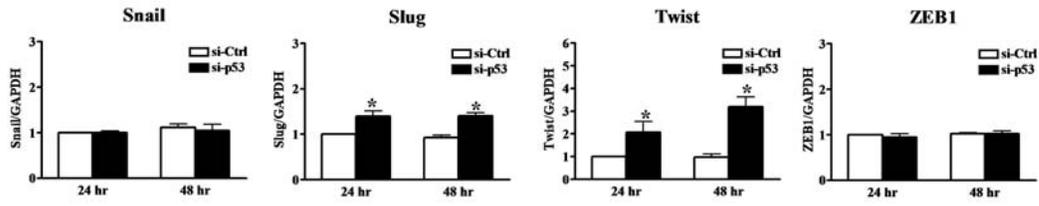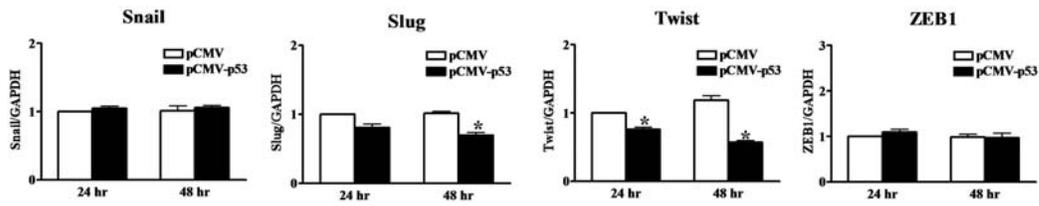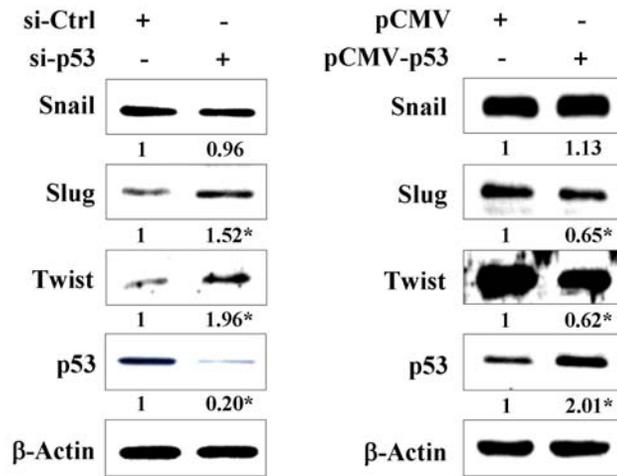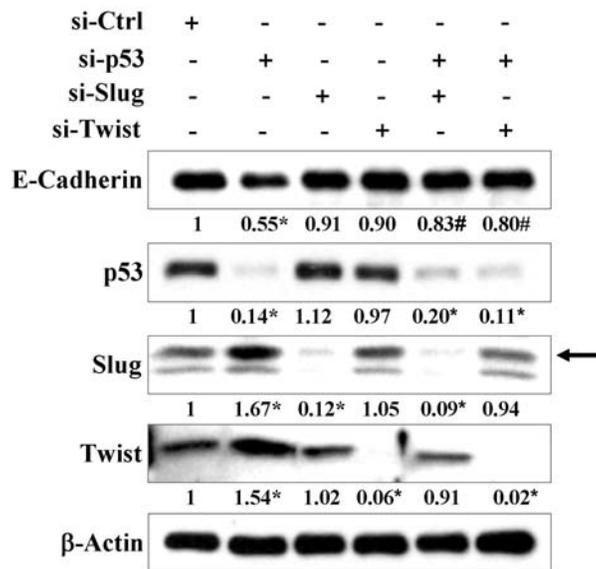
**Figure 3. 9 Inhibition of p53 downregulates E-cadherin by increasing the transcriptional repressors Slug and Twist**

**(A and B)** The mRNA and protein levels of E-cadherin were analyzed by RT-qPCR and western blot in p53-knocked down SBOT3.1 cells and p53-overexpressing MPSC1 cells. **(C)** Cells were seeded on the coverslips and transfected with p53 siRNA (SBOT3.1) or pCMV-p53 (MPSC1) for 72 hr. The expression of E-cadherin (green) and p53 (red) was examined by immunofluorescence staining. Nuclei were stained with Hoechst 33258 (blue). Scale bar represents 100 μm. **(D)** The mRNA levels of Snail, Slug, Twist and ZEB1 were analyzed by RT-qPCR in p53-knocked down SBOT3.1 cells and p53-overexpressing MPSC1 cells. **(E)** The protein levels of Snail, Slug and Twist were analyzed by western blot in p53-knocked down SBOT3.1 cells and p53-overexpressing MPSC1 cells. The levels of E-cadherin protein were examined after transfection of the cells with p53 siRNA or pCMV-p53 for 72 hr. The protein levels of Snail, Slug and Twist were examined after transfection of the cells with p53 siRNA or pCMV-p53 for 48 hr. **(F)** SBOT3.1 cells were transfected with p53 siRNA alone or in conjunction with Slug or Twist siRNA for 72 hr, and the protein levels of E-cadherin were analyzed by western blot. Arrow indicates the band of Slug. Numbers under the western blots represent the densitometry quantifications. The results of RT-qPCR are expressed as the mean ± SEM of at least three independent experiments. *$p < 0.05$ compared with si-Ctrl or pCMV.
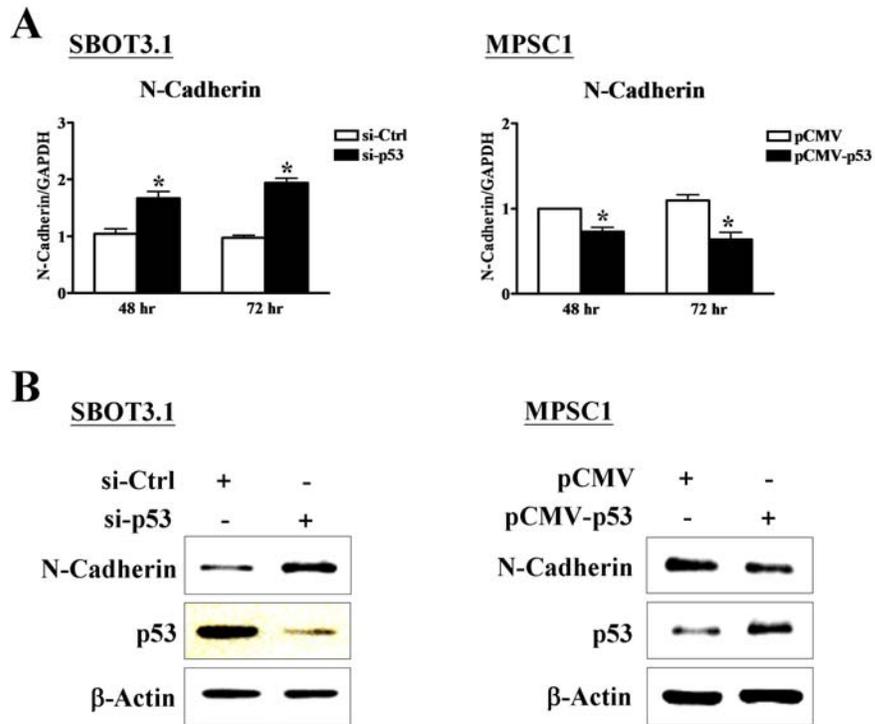
**Figure 3. 10 Inhibition of p53 upregulates N-cadherin**

**(A and B)** The mRNA and protein levels of N-cadherin were analyzed by RT-qPCR and western blot in p53-knocked down SBOT3.1 cells and p53-overexpressed MPSC1 cells. RT-qPCR results are expressed as mean ± SEM of at least three independent experiments. *$p<0.05$ compared with si-Ctrl or pCMV.
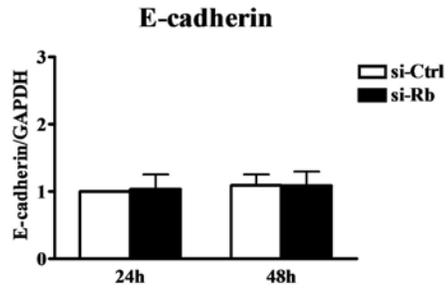
**Figure 3. 11 Inhibition of Rb did not affect the level of E-cadherin in SBOT3.1 cells**

SBOT3.1 cells were transfected with Rb siRNA for 24 and 48 hr and the mRNA level of E-cadherin was analyzed by RT-qPCR. RT-qPCR results are expressed as mean ± SEM of at least three independent experiments.
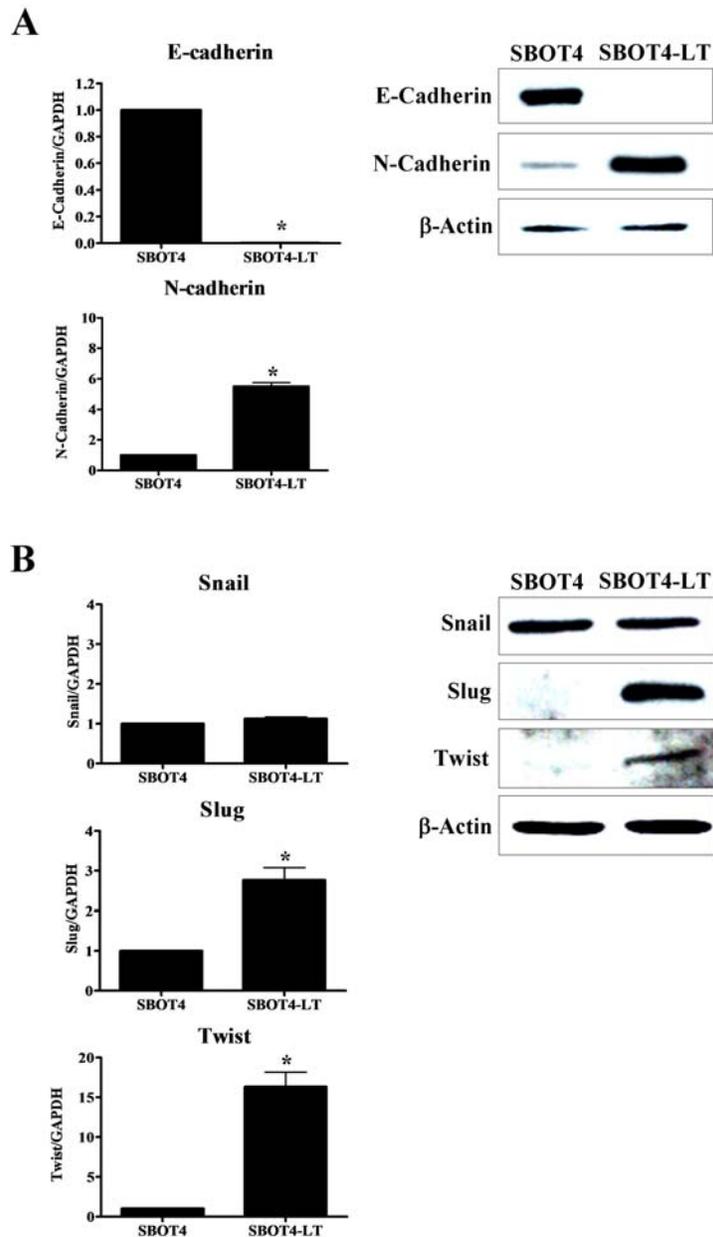
**Figure 3. 12 SV40 LT decreases E-cadherin and increases N-cadherin, Slug and Twist in SBOT4 cells**

**(A)** The mRNA and protein levels of E-cadherin in SBOT4 and SBOT4-LT cells were analyzed by RT-qPCR (left panel) and western blot (right panel). **(B)** The mRNA and protein levels of the E-cadherin transcriptional repressors, Snail, Slug and Twist, in SBOT4 and SBOT4-LT cells were analyzed by RT-qPCR (left panel) and western blot (right panel). RT-qPCR results are expressed as mean ± SEM of three different passages of cells. *p<0.05 compared with SBOT4.
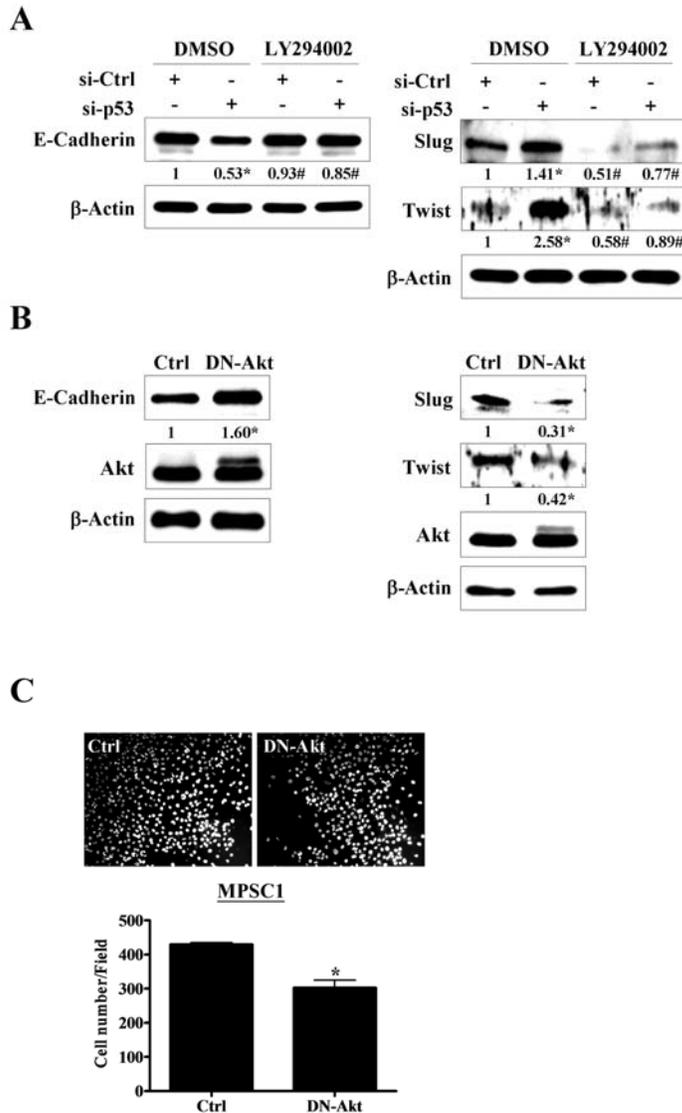
**Figure 3. 13 Akt activation is required for inhibition of the p53-mediated down-regulation of E-cadherin**

**(A)** SBOT3.1 cells transfected with control or p53 siRNA were treated with 10 μM LY294002, after which the protein levels of E-cadherin (after 72 hr of transfection), Slug and Twist (after 48 hr of transfection) were examined by western blot. **(B)** MPSC1 cells were transfected with DN-Akt, and the protein levels of E-cadherin (after 72 hr of transfection), Slug and Twist (after 48 hr of transfection) were examined by western blot. **(C)** MPSC1 cells were transfected with DN-Akt for 48 hr, and the cell invasion of the cells was analyzed by culturing the cells in Matrigel-coated transwells. Numbers under the western blots represent the densitometry quantifications. The results are expressed as the mean ± SEM of at least three independent experiments. *p<0.05 compared with Ctrl.

**A**

SBOT3.1

| | | |
|---|---|---|
| si-Ctrl | + | - |
| si-EC | - | + |

E-Cadherin

1     0.49*

β-Actin

si-Ctrl      si-EC



**B**

SBOT3.1

| | | | | |
|---|---|---|---|---|
| si-Ctrl | + | - | + | - |
| si-p53 | - | + | - | + |
| pIRES | + | + | - | - |
| pIRES-EC | - | - | + | + |

E-Cadherin

1   0.41*   3.89#   3.68#

p53

1   0.13*   1.09   0.15*

β-Actin

si-Ctrl-pIRES    si-p53-pIRES
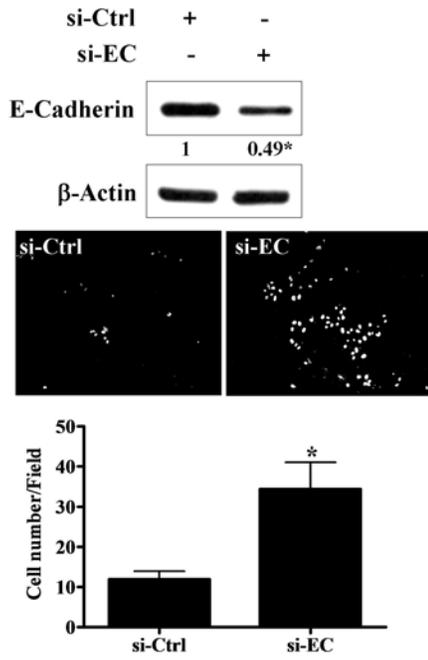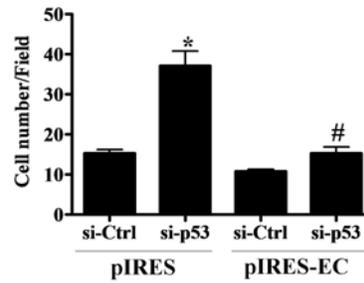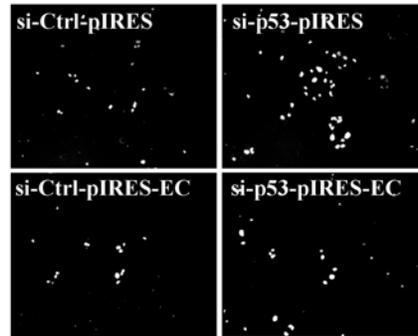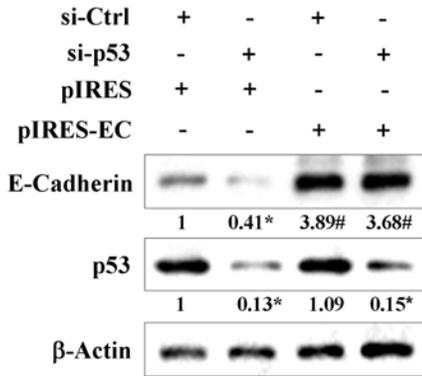
si-Ctrl-pIRES-EC    si-p53-pIRES-EC
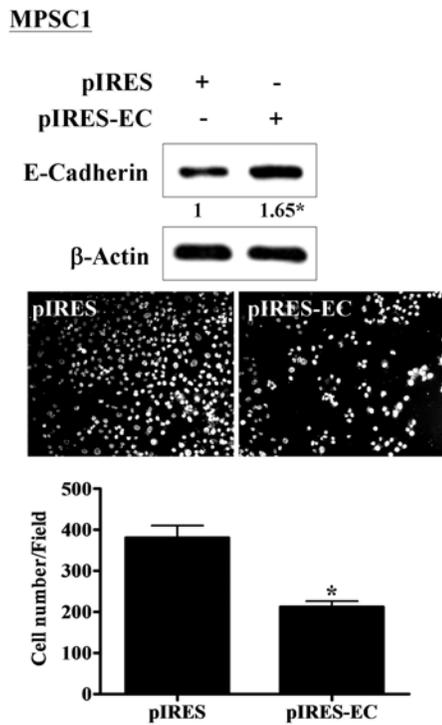
**Figure 3. 14 E-cadherin negatively regulates the cell invasion of SBOT3.1 and MPSC1 cells**

**(A)** SBOT3.1 cells were transfected with E-cadherin siRNA (si-EC) for 48 hr and then seeded into Matrigel-coated transwells. The efficiency of E-cadherin knockdown was examined by western blot (upper panel). The middle panel shows representative fluorescence images of the invasion assay; the lower panel shows a summary of the statistical analysis. **(B)** SBOT3.1 cells were transfected with p53 siRNA or in conjunction with pIRES-EC. The efficiency of p53 knockdown and E-cadherin overexpression were analyzed by western blot. The cell invasion was analyzed by Matrigel-coated transwells. **(C)** MPSC1 cells were transfected for 48 hr with control vector (pIRES) or vector containing full-length E-cadherin cDNA (pIRES-EC) and then seeded into Matrigel-coated transwells. The expression levels of E-cadherin protein were analyzed by western blot (upper panel). The middle panel shows representative fluorescence images of the invasion assay; the lower panel shows a summary of the statistical analysis. Numbers under the western blots represent the densitometry quantifications. The results are expressed as the mean ± SEM of at least three independent experiments. *p<0.05 compared with si-Ctrl or pIRES. [#]p<0.05 compared with pIRES-si-p53.
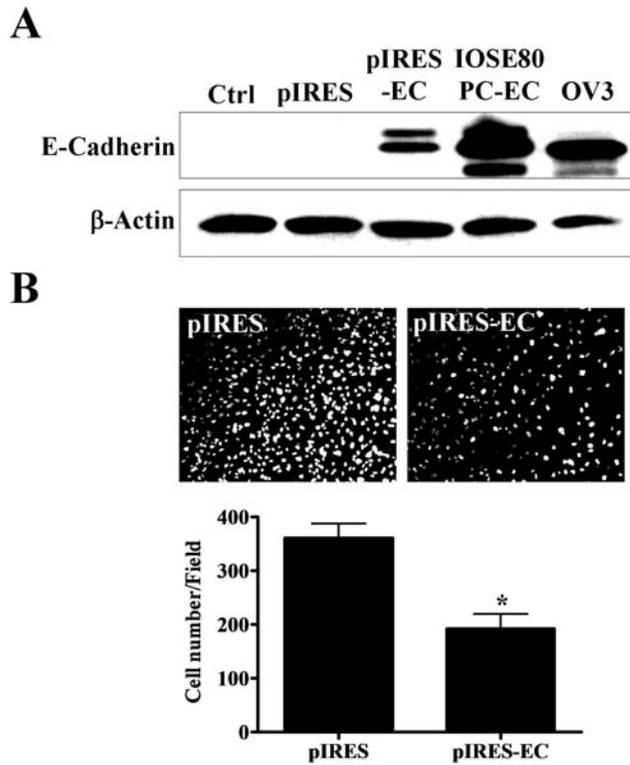
**Figure 3. 15 Enhanced expression of E-cadherin diminishes the cell invasion of SBOT4-LT cells**

**(A)** SBOT4-LT cells were transfected for 48 hr with control vector (pIRES) or vector containing a full-length wild-type E-cadherin cDNA (pIRES-EC), and the protein level of E-cadherin was analyzed by western blot. IOSE80PC-EC, an E-cadherin overexpressing SV40 LT antigen immortalized ovarian surface epithelial cells created with the same expressing vector and cells from an ovarian cancer cell line, OVCAR3, were used as positive controls. **(B)** After 48 hr transfection, cell invasion was examined in Matrigel-coated transwells. Results are expressed as mean ± SEM of four independent experiments. *p<0.05 compared with pIRES.

# Chapter 4. Inhibition of p53 represses E-cadherin expression by increasing DNA methyltransferase 1 and promoter methylation in serous borderline ovarian tumor cells

## 4.1 Introduction

Progression of the transition from benign tumors to metastatic tumors is characterized by the acquisition of the ability to overcome cell-cell adhesion and invade surrounding tissue. E-cadherin is localized to the surface of epithelial cells in regions of cell-cell contacts known as adherens junctions, and it maintains cell polarity and normal epithelial structure (216, 217). An early event of epithelial ovarian carcinogenesis is characterized by abnormal epithelial differentiation; in contrast to most epithelial cancers that down-regulation of E-cadherin correlates with the tumor progression, primary differentiated ovarian cancers express abundant E-cadherin. Although during the progression of cancer complete loss of E-cadherin expression is uncommon, reduced E-cadherin staining is often detected in late-stage ovarian cancer and in ascites-derived tumor cells (218). Repression of E-cadherin expression and mutations in the E-cadherin gene are prevalent in many epithelial cancers (219). Because mutations of the E-cadherin gene are very rare in ovarian carcinoma (152), alterations in E-cadherin expression that are associated with disease progression may be attributed to two mechanisms: down-regulation of transcription by inhibitory transcription factors and hypermethylation of the promoter (220).

DNA methylation is an epigenetic event in which a methyl group is added to the fifth carbon position of a cytosine residue that mostly occurs in CpG sites (221). To date, three known classes of catalytically active DNA methyltransferases (DNMTs), DNMT1, DNMT3a, and DNMT3b, have been cloned and characterized (222). DNMT1 is essential for

maintaining DNA methylation patterns in proliferating cells (223). DNMT3a and DNMT3b are required for *de novo* methylation during embryonic development (224). In human cancers, aberrant DNA methylation is one of the most consistent epigenetic mechanisms that regulate gene expression (225). The E-cadherin 5' transcriptional start site contains a dense CpG island. In different human cancers, down-regulation of E-cadherin is linked to aberrant methylation of this CpG island (226, 227). Inhibition of DNMT1 using siRNA or the DNMT inhibitor 5-Aza-2′-deoxycytidine (5-Aza-dC) is sufficient to reverse promoter methylation in many tumor suppressor genes, including E-cadherin (228, 229), and constitutive expression of DNMT1 is sufficient to induce tumorigenic transformation (230).

Serous borderline ovarian tumors (SBOT) are noninvasive neoplasms that are considered to be distinct entities and give rise to invasive low-grade serous carcinomas (LGSC), which have a relatively poor prognosis (200, 231). The mutation of p53 is the most common genetic alteration in high-grade invasive ovarian carcinomas, whereas it is rare in low-grade invasive carcinomas and borderline tumors (60, 210). Recently, we have shown that inhibition of p53 induces invasiveness in SBOT cells by down-regulating E-cadherin expression. Down-regulation of E-cadherin is mediated by increases in PI3K/Akt signaling and expression of transcriptional repressors (232). Deletion of p53 increases DNMT1 mRNA and protein levels, suggesting relief from p53-mediated DNMT1 repression (233). We therefore asked whether p53 is involved in the regulation of DNMT1 expression in SBOT cells and whether DNMT1-mediated epigenetic repression of E-cadherin may contribute to the transition from noninvasive borderline to invasive ovarian carcinomas.

## 4.2 Material and methods

### Cell culture

The SBOT3.1 and SV40 large T antigen-infected SBOT4-LT cells were grown in a 1:1

(v/v) mixture of M199/MCDB105 media (Sigma, Oakville, ON) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories Inc., Logan, UT). The MPSC1 cell line, which was established from a LGSC (provided by Dr. Ie-Ming Shih, Department of Pathology, Johns Hopkins Medical Institutions, Baltimore, MD), was maintained in RPMI 1640 (Invitrogen, Burlington, ON) supplemented with 10% FBS (208). Cultures were maintained at 37°C in a humidified atmosphere of 5% $CO_2$ in air.

**Western blots**

Cells were lysed, and protein concentrations were analyzed with a protein assay kit using BSA standards according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA). Equal amounts of cell lysates were separated by SDS-PAGE and transferred to PVDF membranes. After blocking with TBS containing 5% non-fat dry milk, the membranes were incubated overnight at 4°C with anti-E-cadherin (BD Biosciences, Mississauga, ON), anti-DNMT1 (Abnova, Taipei, Taiwan) and anti-p53 (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies, followed by incubation with a HRP-conjugated secondary antibody. Immunoreactive bands were detected using an enhanced chemiluminescence (ECL) kit. The membrane was stripped with stripping buffer and re-probed with an anti-β-actin antibody as a loading control.

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

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription was performed with 3 µg RNA, random primers and M-MLV reverse transcriptase (Promega, Madison, WI). The primers used for SYBR Green reverse transcription-qPCR (RT-qPCR) were as follows: E-cadherin, 5'-ACA

GCC CCG CCT TAT GAT T-3' and 5'-TCG GAA CCG CTT CCT TCA-3'; DNMT1, 5'-GAG CCA CAG ATG CTG ACA AA-3' and 5'-TGC CAT TAA CAC CAC CTT CA-3'; DNMT3a, 5'-CAA TGA CCT CTC CAT CGT CAA C-3' and 5'-CAT GCA GGA GGC GGT AGA A-3'; DNMT3b, 5'-CCA TGA AGG TTG GCG ACA A-3' and 5'-TGG CAT CAA TCA TCA CTG GAT T-3'; p53, 5'-GTT CCG AGA GCT GAA TGA GG-3' and 5'-TCT GAG TCA GGC CCT TCT GT-3'; Rb, 5'-TCC CAT GGA TTC TGA ATG TG-3' and 5'-CCT TCT CGG TCC TTT GAT TG-3'; and GAPDH, 5'-GAG TCA ACG GAT TTG GTC GT-3' and 5'-GAC AAG CTT CCC GTT CTC AG-3'. RT-qPCR was performed using the ABI Prism 7000 Sequence Detection System equipped with a 96-well optical reaction plate. All RT-qPCR experiments were run in triplicate, and a mean value was used to determine mRNA levels. Relative quantification of mRNA levels was performed using the comparative Ct method with GAPDH as the reference gene and the formula $2^{-\Delta\Delta Ct}$.

**Methylation-specific PCR (MSP)**

Detection of E-cadherin promoter methylation was performed as previously described (234). Briefly, genomic DNA was extracted from the cells, and unmethylated cytosine bases were converted to uracil using the Imprint DNA Modification kit (Sigma). The following primers were used for the amplification of a 116-bp fragment corresponding to the E-cadherin methylated promoter region: forward primer, 5'-TTA GGT TAG AGG GTT ATC GCG T-3' and reverse primer, 5'-TAA CTA AAA ATT CAC CTA CCG AC-3'. The following primers were used for the amplification of a 97-bp fragment corresponding to the unmethylated promoter region: forward primer, 5'-TAA TTT TAG GTT AGA GGG TTA TTG T-3' and reverse primer, 5'-CAC AAC CAA TCA ACA ACA CA-3'. The MSP was performed using the following cycling conditions: denaturation at 95°C for 5 min, followed

by 40 cycles of 95°C for 30 s, 57°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 5 min. MSP bands were detected by electrophoresis in a 2% agarose gel stained with ethidium bromide.

**Invasion assay**

The invasion assay was performed in Boyden chambers (209). Filters (8-μm pore size, 24 wells, BD Biosciences, Mississauga, ON) were coated with 1 mg/mL growth-factor-reduced Matrigel (BD Biosciences). Cells in M199/MCDB105 media supplemented with 0.1% FBS were incubated for 48 hr against a gradient of 10% FBS. Cells that penetrated the membrane were fixed with cold methanol and stained with Hoechst 33258, and the number of nuclei stained with Hoechst 33258 was counted using Northern Eclipse 6.0 software from Empix Imaging (Mississauga, ON, Canada). Each individual experiment consisted of triplicate inserts, and five microscopic fields were counted per insert.

**Small interfering RNA and transfection**

To inactivate p53, E-cadherin and DNMT1, cells were transfected with p53, E-cadherin and DNMT1 siRNA (Dharmacon Research, Inc., Lafayette, CO) using Lipofectamine RNAiMAX (Invitrogen). The non-targeting siRNA (Dharmacon) was used as a transfection control. Myc-tagged dominant-negative Akt (DN-Akt) (Upstate, Billerica, MA) was transfected into cells using Lipofectamine 2000.

**Statistical analysis**

Results are presented as the mean ± SEM of at least three independent experiments. Statistical evaluation was conducted using a *t*-test for paired data. Multiple comparisons were

analyzed first by one-way ANOVA, followed by Tukey's multiple comparison tests. A significant difference was defined as $p<0.05$.

## 4.3 Results

**DNMT1 expression is increased in SV40 large T antigen (LT)-infected SBOT cells**

To investigate the effect that p53 has on DNMT1 expression in SBOT cells, we first examined DNMT1 expression in SV40 LT-infected SBOT cells, SBOT4-LT, which we recently generated (232). RT-qPCR results demonstrated that DNMT1 mRNA levels were increased in SBOT4-LT cells compared to parental SBOT4 cells, whereas DNMT3a and DNMT3b did not vary significantly (Figure 4.1A). Consistent with mRNA levels, DNMT1 protein levels also increased in SBOT4-LT cells, as determined by western blot (Figure 4.1B).

**5-Aza-dC restores E-cadherin expression and decreases cell invasion in SBOT4-LT and MPSC1 cells**

Recently, we showed that inhibition of p53 caused E-cadherin to be down-regulated in SBOT4-LT cells and in MPSC1 (invasive LGSC cells) (232). RT-qPCR results showed that DNMT1 mRNA levels were higher in SBOT4-LT and MPSC1 cells compared to the spontaneously immortalized permanent cell line SBOT3.1. Consistent with mRNA levels, DNMT1 protein levels were also higher, as determined by western blot (Figure 4.2A). Therefore, we tested the possibility that E-cadherin promoter inactivation occurred via inhibition of p53-induced hypermethylation. E-cadherin mRNA and protein levels were examined after treating cells with the DNMT1 inhibitor, 5-Aza-2′-deoxycytidine (5-Aza-dC). As shown in Figure 4.2B, 5-Aza-dC restored E-cadherin mRNA levels in a dose-dependent

manner in both SBOT-4LT and MPSC1 cells. Consistent with this result, E-cadherin protein levels also increased in cells treated with 5-Aza-dC (Figure 4.2C). Additionally, methylation-specific PCR analysis revealed that CpG island 1 from the E-cadherin promoter is methylated in SBOT4-LT and MPSC1 cells and that this methylation was abolished by treatment with 5-Aza-dC (Figure 4.2D). Because E-cadherin is an important invasion repressor in SBOT cells, we examined whether restoration of E-cadherin levels after treatment with 5-Aza-dC regulates cell invasion. As shown by the invasion assay, SBOT4-LT and MPSC1 cells were invasive, and their invasiveness was significantly inhibited by treatment with 5-Aza-dC (Figure 4.2E). In addition, only high concentrations (20 μM) of 5-Aza-dC slightly inhibited SBOT4-LT and MPSC1 cell proliferation, as determined by counting cells using trypan blue (Figure 4.3).

**E-cadherin promoter methylation is mediated by DNMT1 in SBOT4-LT and MPSC1 cells**

To further confirm that the promoter methylation of E-cadherin is mediated by DNMT1, DNMT1 siRNA was used to knockdown the endogenous expression of DNMT1 in SBOT4-LT and MPSC1 cells. As shown in Figure 4.4A, the DNMT1 mRNA level was decreased after treatment with DNMT1 siRNA. Conversely, the mRNA level of E-cadherin was increased by treatment with DNMT1 siRNA. Western blot showed similar results for the protein level (Figure 4.4B). Additionally, DNMT1 siRNA abolished E-cadherin promoter methylation (Figure 4.4C). Moreover, the invasiveness of SBOT4-LT and MPSC1 cells were significantly inhibited by DNMT1 siRNA treatment which was reversed in combination with E-cadherin siRNA (Figure 4.4D). These results indicated that DNMT1 siRNA-inhibited SBOT4-LT and MPSC1 cell invasion was mediated by restoring the expression of

E-cadherin.

**Inhibition of p53, but not Rb, increases DNMT1 in SBOT cells**

It is well known that SV40 LT inhibits both p53 and Rb. To further discriminate between the effects of p53 and Rb on DNMT1 expression, we knocked down endogenous p53 or Rb and examined the expression of DNMT1 in SBOT3.1 cells. As shown in Figure 4.5A, p53 and Rb siRNA significantly knocked down p53 and Rb mRNA levels, respectively. Interestingly, only p53 siRNA increased DNMT1 mRNA levels, whereas DNMT1 mRNA levels were not affected by Rb siRNA treatment. DNMT3a and DNMT3b mRNA levels were not changed after treatment with either p53 or Rb siRNA in SBOT3.1 cells (Figure 4.5B). Western blot showed that p53 inhibition led to an increase in DNMT1 protein levels (Figure 4.5C). To further confirm that DNMT1 expression induced via the inhibition of p53 contributes to methylation of the E-cadherin promoter, SBOT3.1 cells were treated with p53 siRNA, and methylation of the E-cadherin promoter was examined using methylation-specific PCR. As shown in Figure 4.5D, E-cadherin promoter methylation increased following p53 siRNA treatment.

**The PI3K/Akt pathway mediates the up-regulation of DNMT1 through p53 inhibition**

We have shown that p53 inhibition increases the expression of the catalytic subunit of phosphatidylinositol 3-kinase (*PIK3CA*, p110α) and Akt activation (232). Therefore, we investigated whether the PI3K/Akt pathway is involved in p53-mediated regulation of DNMT1 expression. SBOT4-LT and MPSC1 cells were transfected with PIK3CA siRNA and Myc-tagged dominant-negative Akt (DN-Akt), and DNMT1 mRNA and protein levels were examined. Treatment with PIK3CA siRNA significantly knocked down PIK3CA

mRNA levels (Figure 4.6). DNMT1 mRNA and protein levels were down-regulated in SBOT4-LT and MPSC1 cells treated with the PIK3CA siRNA (Figure 4.7A). In addition, cells transfected with DN-Akt also had lower DNMT1 mRNA and protein levels (Figure 4.7B). When SBOT3.1 cells were treated with a combination of PIK3CA siRNA and p53 siRNA, the p53 siRNA-mediated up-regulation of DNMT1 mRNA and protein levels was diminished (Figure 4.7C).

**DNMT1-mediated promoter methylation is required for the inhibition of p53-mediated down-regulation of E-cadherin**

To further investigate whether DNMT1 is required in p53-mediated down-regulation of E-cadherin, SBOT3.1 cells were treated with p53 siRNA, DNMT1 siRNA, or both, and methylation of the E-cadherin promoter was examined. Methylation-specific PCR showed that p53 siRNA caused an increase in promoter methylation, whereas DNMT1 siRNA caused a decrease in promoter methylation compared to control cells. Interestingly, the increase in promoter methylation caused by p53 siRNA was abolished by co-treatment with DNMT1 siRNA (Figure 4.8A). p53 siRNA caused a decrease in E-cadherin protein levels, and this decrease was diminished by co-treatment with DNMT1 siRNA, as determined by western blot (Figure 4.8B). However, in cells treated with DNMT1 siRNA, there were no significant increases in E-cadherin protein levels. Moreover, cells transfected with p53 siRNA in combination with DNMT1 siRNA diminished p53 siRNA-induced cell invasion, as determined using an invasion assay (Figure 4.8C). Taken together, these results indicate that the inhibition of p53-induced down-regulation of E-cadherin is partly mediated by promoter methylation by DNMT1 and is associated with cell invasion in SBOT cells.

## 4.4 Discussion

In ovarian cancer, aberrant methylation of multiple CpG islands is a frequent event compared with normal ovarian surface epithelium, and inactivation of several genes, including tumor suppressor genes, by aberrant methylation of CpG islands has been documented (235, 236). Recently, we showed that inhibition of p53 increases SBOT cell invasion by increasing the transcriptional repressors Slug and Twist, leading to the down-regulation of E-cadherin (232). However, it is unknown whether changes in promoter methylation are involved in the inhibition of p53-induced down-regulation of E-cadherin. In the present study, we found that DNMT1 levels, but not DNMT3a or DNMT3b levels, increased in SBOT cells after p53 was inhibited. The activation of the PI3K/Akt pathway is required for p53 inhibition-induced DNMT1 expression. Furthermore, the increase in DNMT1 contributes to E-cadherin promoter methylation leading to the down-regulation of E-cadherin and SBOT cell invasion. Previously, we have shown that p53 binds directly to the *PIK3CA* promoter and inhibits its activity in ovarian cancer cells (206). In addition, p53 inhibition increases the expression of the *PIK3CA* and Akt activation in SBOT cells (232). Taken together, our results indicate that p53 acts as a tumor suppressor in the progression from SBOT to invasive ovarian carcinoma by regulating E-cadherin expression through PI3K/Akt-mediated transcriptional and epigenetic machineries (Figure 4.9).

Previous studies have compared epigenetic aberrations in benign, borderline and malignant invasive ovarian tumors. The results from these studies showed that borderline tumors have a methylation profile that is in between that of benign and malignant invasive tumors. In addition, data on aberrant epigenetic alterations suggest that some invasive ovarian cancers may originate through an accumulation of methylation events at specific genes in noninvasive precursor lesions (237-239). Interestingly, these studies also support the

dualistic model for ovarian carcinogenesis (22). However, the detailed mechanism underlying gene methylation and ovarian cancer progression requires further investigation.

Mutation of p53 is the most common genetic alteration in high-grade invasive ovarian carcinomas, whereas it is rare in low-grade invasive carcinomas and borderline tumors (60). We recently showed that the inhibition of p53 activity, either by SV40 LT or p53 siRNA, increased the invasiveness of SBOT cells (232). Additionally, a microarray study showed that expression profiles from low-grade invasive carcinomas lack the enhanced p53 expression and signaling activity observed in SBOT cells (54). These results indicate that the progression from SBOT to low-grade invasive carcinoma involves the attenuation of p53 signaling. p53 is a very important tumor suppressor gene and is reported to be abnormal in most human cancers (240). p53 binding sites have been identified in the 5'-flanking region and exon 1 of the human DNMT1 genomic locus (233). In addition, several p53 binding sites are also found in the 5' region of the mouse DNMT1 locus (233). Deletion of p53 in human colon carcinoma cells and primary mouse astrocytes results in an increase in DNMT1 mRNA and protein, suggesting that p53-mediates transcriptional repression of this gene (233). Recently, it was shown that activation of the PI3K/Akt pathway increases DNMT1 protein levels (241). In the present study, we found that inhibition of p53 increases DNMT1 expression. In addition, inhibition of the PI3K/Akt pathway diminished the up-regulation of DNMT1 when p53 is inhibited. However, we did not show the direct binding of p53 to the DNMT1 promoter in SBOT cells. Thus, whether the expression of DNMT1 is regulated by direct binding of p53 to the DNMT1 promoter or by p53-mediated up-regulation of PI3K/Akt signaling will need further investigation.

The defining characteristic of epithelial-mesenchymal transition (EMT) is a down-regulation in E-cadherin expression (212). The PI3K/Akt pathway is important in

ovarian carcinogenesis and is implicated in the regulation of cell migration, invasion and EMT (211, 242). It has been shown that PI3K/Akt pathway can interact with other pathways of EMT such as tyrosine kinases, Wnt/β-catenin, RAS, Notch and integrin-linked kinase (ILK) signaling (185). Squamous cell carcinoma cell lines exhibit constitutive activation of Akt, which down-regulates the expression of E-cadherin and promotes tumorigenicity and invasiveness (207). Such findings indicate that activation of the PI3K/Akt axis coupled with the down-regulation of E-cadherin expression may be important in promoting tumorigenesis.

DNMT1 is the most extensively studied and most abundant DNMT; it is thought to be responsible for replicating methylation patterns after DNA synthesis (243). DNMT1 knockouts are resistant to colorectal tumorigenesis, and antisense knockdowns of DNMT1 reverse tumorigenesis *in vitro* and *in vivo* (244-246). In ovarian cancer, differential DNMT gene expression has been examined, and it has been suggested that alterations in DNMT expression might contribute to the CpG island methylation phenotype (247). RNAi-mediated down-regulation of DNMT1 protein expression restores the expression of some inactive genes, such as *RASSF1A* and *HIN-1*, due to a decrease in the methylation levels of these genes in an ovarian cancer cell line (248). We found that the increase in DNMT1 expression caused by p53 inhibition leads to the down-regulation of E-cadherin expression, and contributes to the invasiveness of SBOT cells. Our results may also explain the higher level of DNMT1 in the high-grade serous ovarian cancer cells which frequently possesses mutant p53 when compared to the normal ovarian surface epithelial cells (247). Taken together, these results indicate that DNMT1 is required to maintain the aberrant methylation in cancer cells and that the DNMT1-regulated methylation of these sites is required for the transcriptional silencing of tumor suppressor genes in human cancer.

Ovarian cancer cells with low E-cadherin expression are more invasive (192), and the

absence of E-cadherin expression in ovarian cancer predicts poor patient survival compared to ovarian tumors that express E-cadherin (190). Several studies have shown that DNMT1 is necessary to maintain the methylation of CpG islands in the E-cadherin promoter (229, 249-251). However, recent studies in endometrial epithelial carcinoma and choriocarcinoma cell lines have shown that the siRNA-mediated down-regulation of the individual DNMT genes did not cause an increase in E-cadherin expression; however, concurrent knockdowns of DNMT3a and DNMT3b induced E-cadherin expression. Furthermore, E-cadherin expression significantly increased after the concomitant knockdown of DNMT1, 3a, and 3b (252, 253). Here, we show that inhibition of p53 leads to an increase in DNMT1 expression and does not alter the expression of DNMT3a or DNMT3b in SBOT cells. These results suggest that epigenetic regulation of E-cadherin by DNMTs might be tissue-specific.

In summary, the results from this study indicate that the inhibition of p53 down-regulates E-cadherin by increasing DNMT1 expression and the subsequent E-cadherin promoter methylation in SBOT cells. This function suggests that p53 acts as an ovarian epithelial tumor suppressor by regulating gene methylation in the progression from SBOT to invasive ovarian carcinoma.
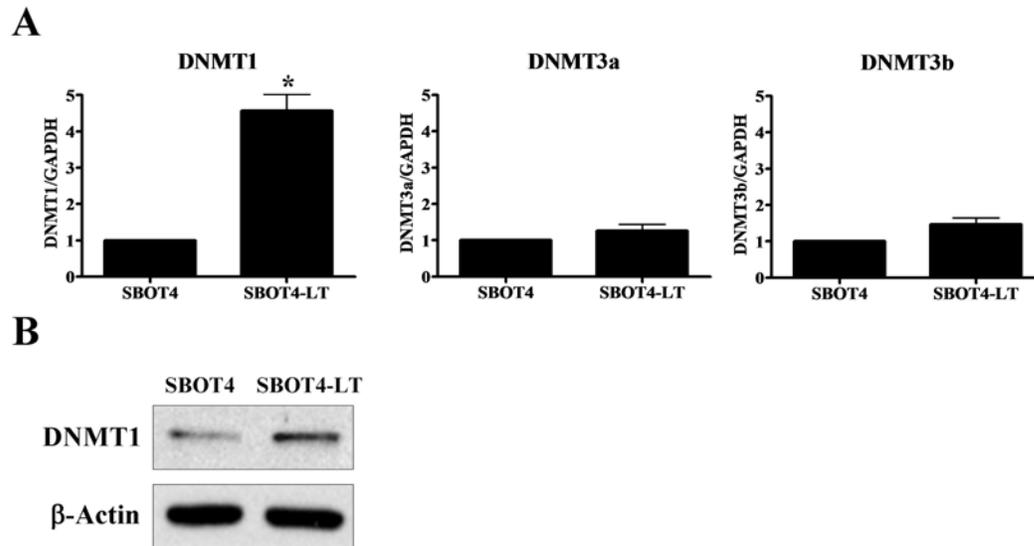
**Figure 4. 1 SV40 large T antigen (LT) increases DNMT1 expression in SBOT cells**
**(A)** Expression levels for three different DNMTs were examined using RT-qPCR in SV40 LT-infected SBOT4 cells (SBOT4-LT). The parent cell line, SBOT4, was used as a control. **(B)** DNMT1 protein levels were examined by western blot in SBOT4-LT cells. The upper panel is representative of the western blot results. The lower panel shows the summarized statistical results. Results are expressed as the mean ± SEM of three different passages of cells. *p<0.05 when compared to SBOT4.

**A**
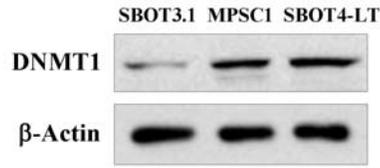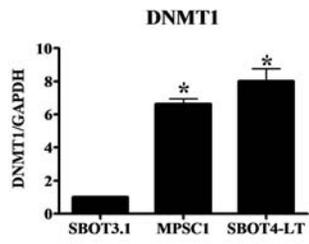


**B**
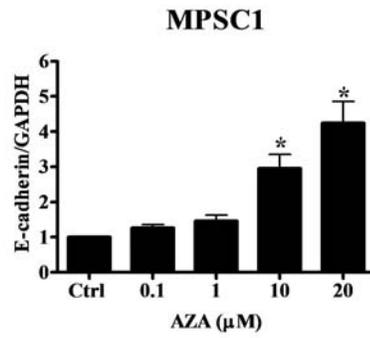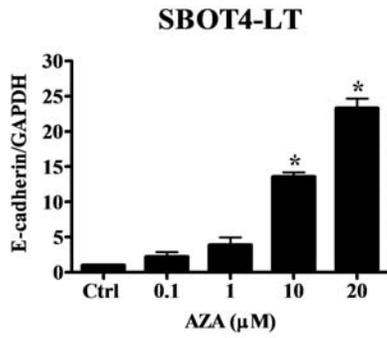


**C**
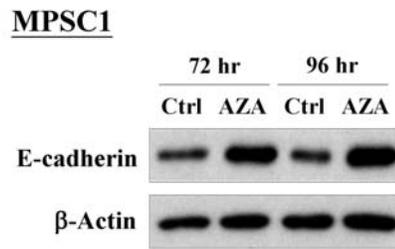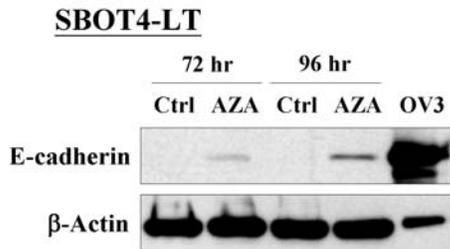


**D**
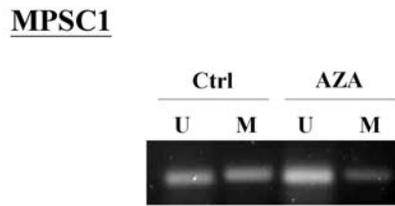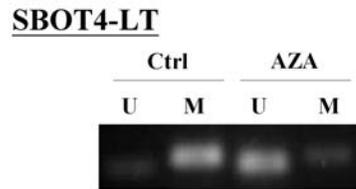
**E**
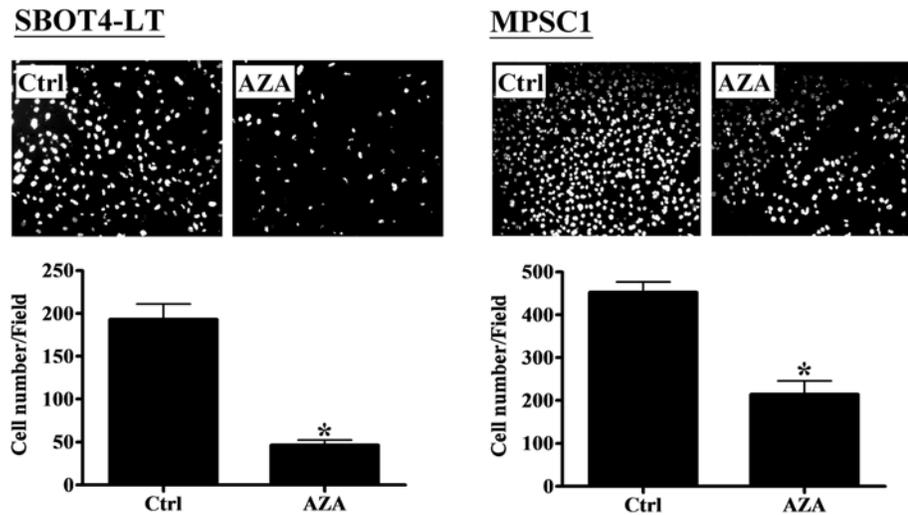


**Figure 4. 2 5-Aza-dC treatment restores E-cadherin expression and decreases cell invasion in SBOT4-LT and MPSC1 cells**

**(A)** DNMT1 mRNA and protein levels in SBOT3.1, SBOT4-LT and MPSC1 cells were examined by RT-qPCR (left panel) and western blot (right panel), respectively. **(B)** Cells were treated with increasing concentrations of 5-Aza-dC for 72 hr. E-cadherin mRNA levels were examined by RT-qPCR. **(C)** Cells were treated with 10 μM 5-Aza-dC for 72 and 96 hr. E-cadherin protein levels were examined by western blot. The ovarian cancer cell line, OVCAR3, was used as a positive control. **(D)** Cells were treated with 10 μM 5-Aza-dC for 72 hr. Methylation of the E-cadherin promoter was examined by methylation-specific PCR. Amplification was performed with primers specific for unmethylated (U) and methylated (M) DNA. E, Cells were treated with 10 μM 5-Aza-dC for 48 hr. Invasiveness was examined using the Matrigel-coated transwell assay. The upper panel shows representative fluorescent images from the invasion assay. The lower panel shows the summarized statistical results. RT-qPCR results expressed as the mean ± SEM of three independent experiments. *p<0.05 when compared to Ctrl.
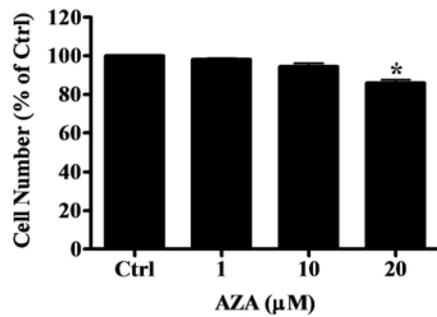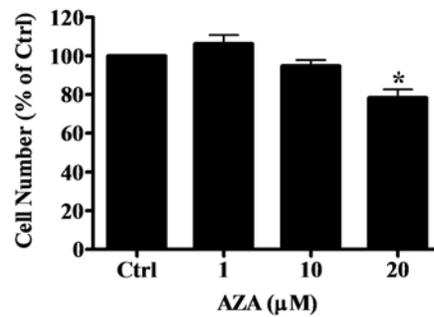
**Figure 4. 3 5-Aza-dC treatment decreases cell proliferation in SBOT-4LT and MPSC1 cells**

Cells were treated with increasing doses of 5-Aza-dC for 96 hr. The number of cells was counted by trypan blue staining. Results are expressed as the mean ± SEM of three independent experiments. *$p < 0.05$ when compared to Ctrl.

**A**

## SBOT4-LT

**DNMT1**



**E-cadherin**
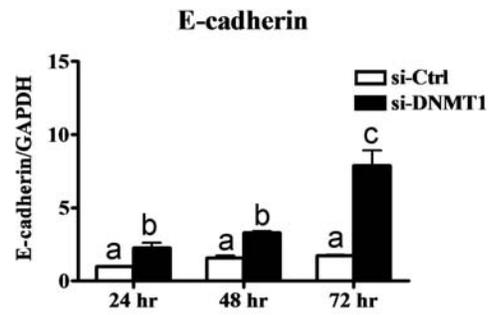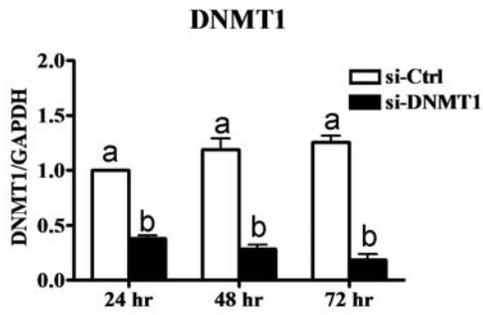


## MPSC1
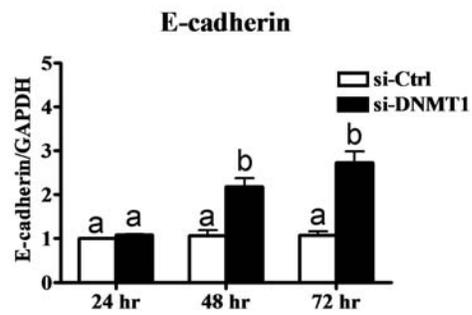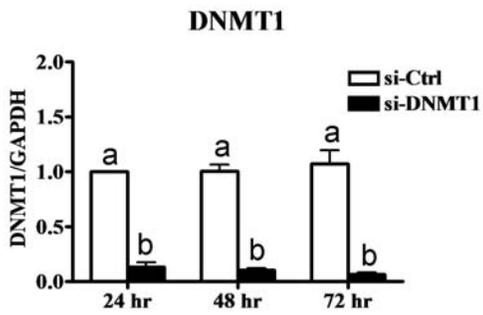
**DNMT1**



**E-cadherin**



**B**
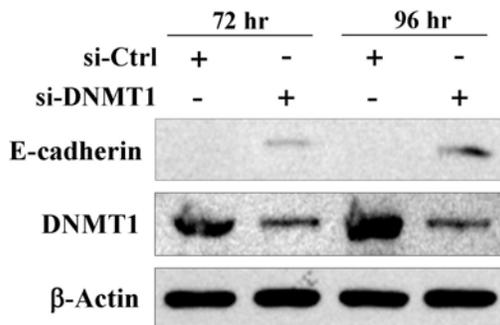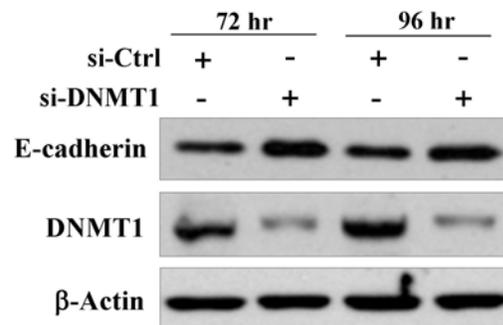
## SBOT4-LT



## MPSC1

**C**

**SBOT4-LT**



**MPSC1**



**D**

**SBOT4-LT**

| si-Ctrl | + | − | − | − |
| si-DNMT1 | − | + | − | + |
| si-E-cadherin | − | − | + | + |

E-cadherin

DNMT1

β-Actin

**MPSC1**

| si-Ctrl | + | − | − | − |
| si-DNMT1 | − | + | − | + |
| si-E-cadherin | − | − | + | + |

E-cadherin

DNMT1

β-Actin





83
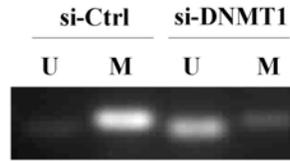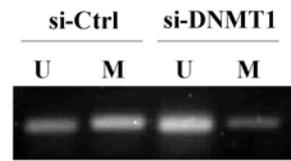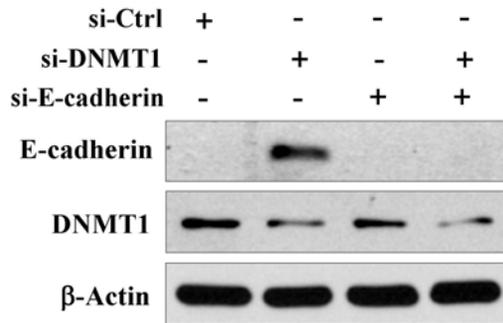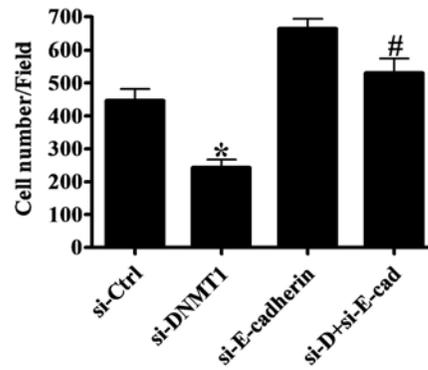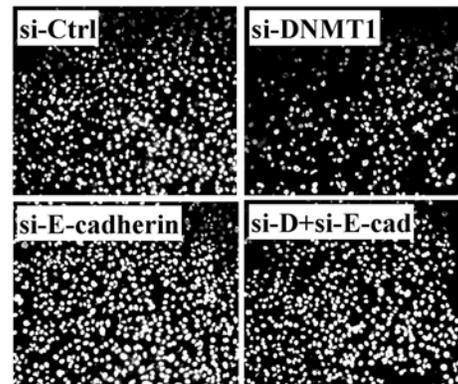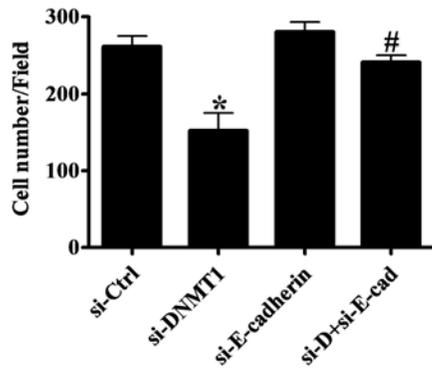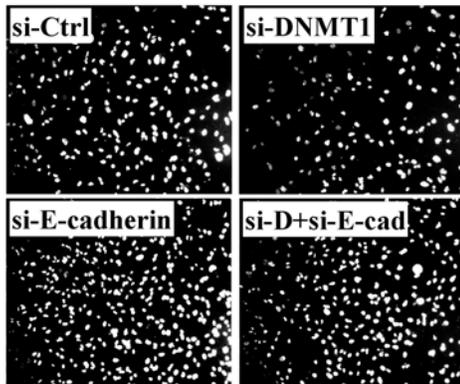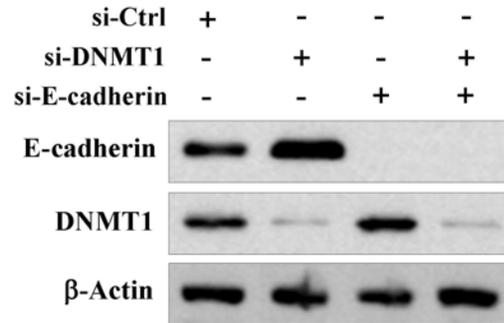
**Figure 4. 4 DNMT1 knockdown restores the expression of E-cadherin and decreases the methylation of its promoter in SBOT4-LT and MPSC1 cells**

**(A)** Cells were transfected with 50 nM non-targeting siRNA (si-Ctrl, white bar) or DNMT1 siRNA (si-DNMT1, black bar) for different amounts of time; the efficiency of DNMT1 siRNA was examined by RT-qPCR (left panel). E-cadherin mRNA levels were examined in DNMT1-knocked down SBOT4-LT cells by RT-qPCR (right panel). Values without a common letter are significantly different (p<0.05). **(B)** Cells were transfected with 50 nM DNMT1 siRNA for 72 and 96 hr. The knockdown of DNMT1 and E-cadherin protein levels were examined by western blot. **(C)** Cells were transfected with 50 nM DNMT1 siRNA for 72 hr. Methylation of the E-cadherin promoter was examined by methylation-specific PCR. Amplification was performed with primers specific for unmethylated (U) and methylated (M) DNA. **(D)** Cells were transfected with non-targeting siRNA (si-Ctrl), DNMT1 (si-DNMT1) siRNA, E-cadherin (si-E-cadherin) siRNA or DNMT1 plus E-cadherin (si-D+si-E-cad) siRNA for 48 hr. Invasiveness was examined using the Matrigel-coated transwell assay. The efficiencies of DNMT1 and E-cadherin siRNA were examined by western blot. The upper panel shows representative fluorescent images from the invasion assay; the lower panel shows the summarized statistical results. Results are expressed as the mean ± SEM of three independent experiments. *p<0.05 when compared to si-Ctrl. [#]p<0.05 when compared to si-DNMT1.
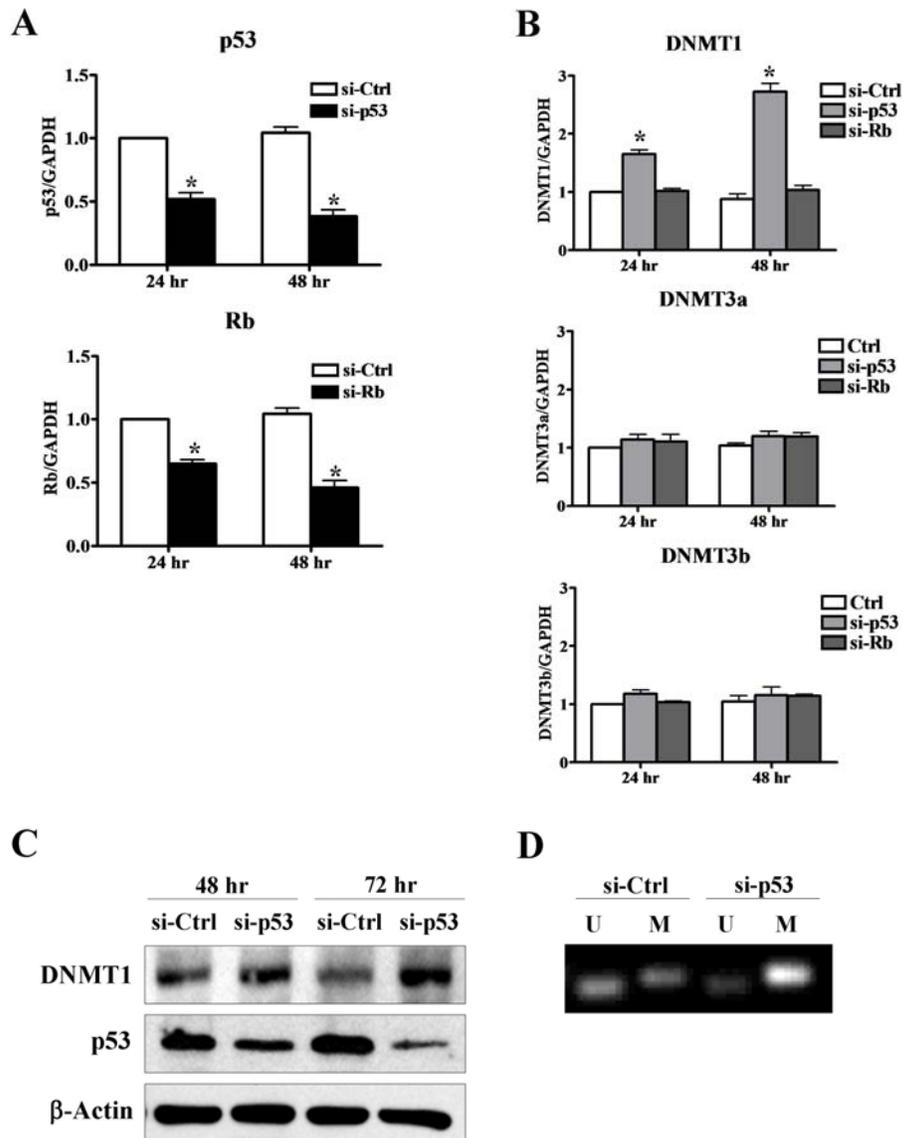
**Figure 4. 5 Knockdown of p53 increases DNMT1 expression in SBOT3.1 cells**

**(A)** SBOT3.1 cells were transfected with 50 nM non-targeting siRNA (si-Ctrl, white bar), p53 (si-p53, black bar) siRNA, or Rb (si-Rb, black bar) siRNA for different amounts of time. The efficiencies of p53 and Rb siRNA were examined by RT-qPCR. **(B)** SBOT3.1 cells were transfected with 50 nM p53 siRNA (si-p53, gray bar) or Rb siRNA (si-Rb, deep gray bar) for different amounts of time. mRNA levels of the three DNMTs were examined by RT-qPCR. **(C)** SBOT3.1 cells were transfected with 50 nM p53 siRNA for 48 and 72 hr. p53 knockdowns and DNMT1 protein levels were examined by western blot. **(D)** SBOT3.1 cells were transfected with 50 nM p53 siRNA for 72 hr. Methylation of the E-cadherin promoter was examined by methylation-specific PCR. Amplification was performed with primers specific for unmethylated (U) and methylated (M) DNA. Results are expressed as the mean ± SEM of three independent experiments. *$p < 0.05$ when compared to si-Ctrl.

85
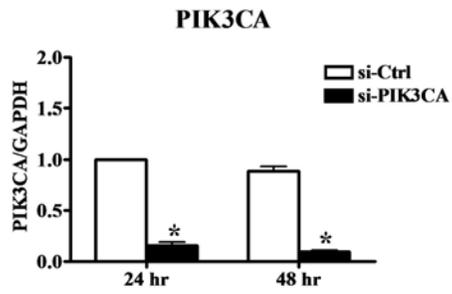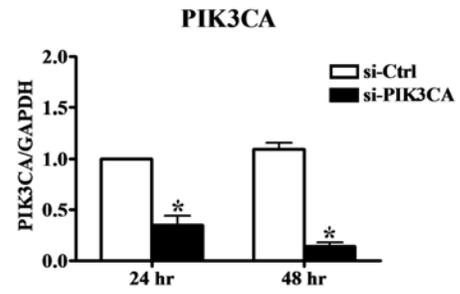
**Figure 4. 6 Knockdown of PIK3CA by siRNA transfection in SBOT4-LT and MPSC1 cells**

The efficiencies of PIK3CA siRNA were examined by RT-qPCR. $*p < 0.05$ when compared to si-Ctrl.
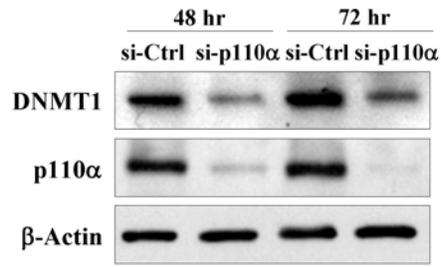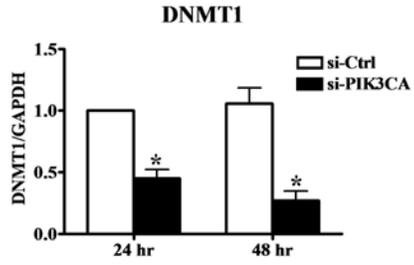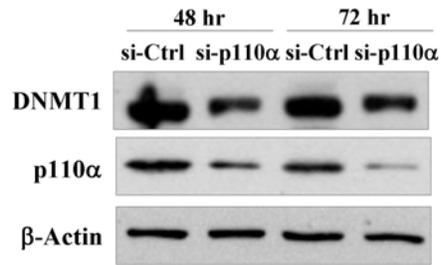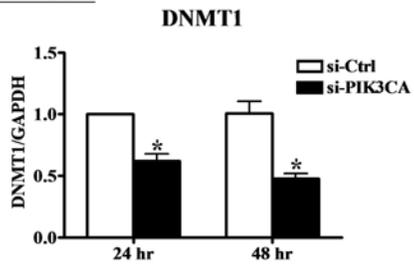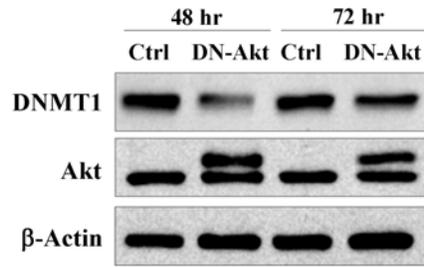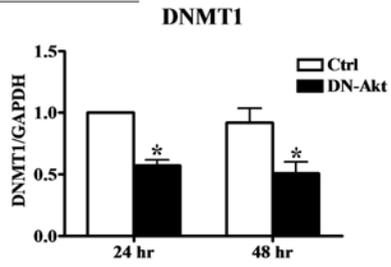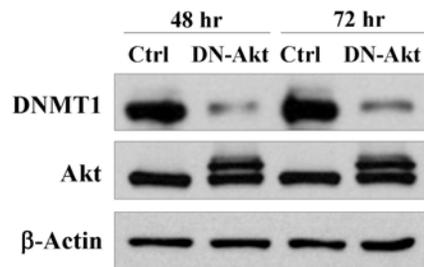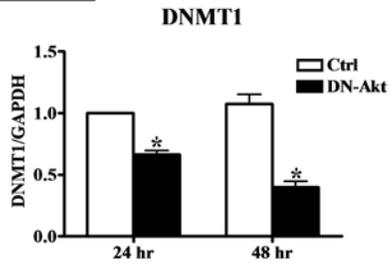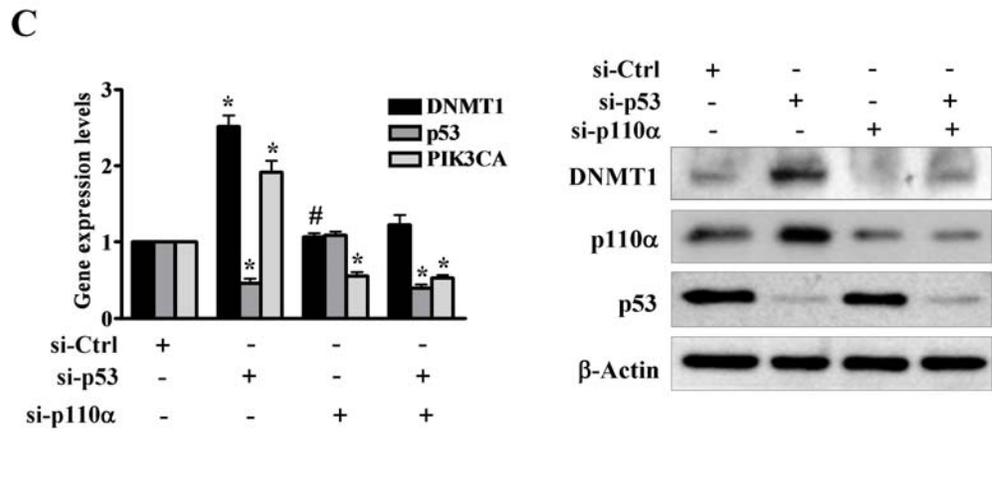
**Figure 4. 7 The PI3K/Akt pathway mediates the up-regulation of DNMT1 induced by p53 inhibition**

**(A)** SBOT4-LT and MPSC1 cells were transfected with 50 nM non-targeting siRNA (si-Ctrl, white bar) or PIK3CA siRNA (si-PIK3CA, black bar). DNMT1 mRNA and protein levels were examined by RT-qPCR (left panel) and western blot (right panel), respectively. **(B)** SBOT4-LT and MPSC1 cells were transfected with 1 μg control vector (white bar) or myc-tagged DN-Akt (black bar). DNMT1 mRNA and protein levels were examined by RT-qPCR (left panel) and western blot (right panel), respectively. The expression of Myc-tagged DN-Akt was verified by western blot using an anti-Akt antibody. **(C)** SBOT3.1 cells were transfected with non-targeting siRNA (si-Ctrl), p53 siRNA (si-p53), PIK3CA siRNA (si-PIK3CA) or a combination of the two. DNMT1 mRNA and protein levels were examined by RT-qPCR (left panel) and western blot (right panel), respectively. *$p<0.05$ when comparing specific genes from the siRNA treatment group to the same genes in the si-Ctrl group. #$p<0.05$ when compared to DNMT1 in the si-p53 group.

**A**

si-Ctrl    si-p53    si-DNMT1    si-p53+si-D
U    M    U    M    U    M    U    M

**B**

si-Ctrl      +    –    –    –
si-p53       –    +    –    +
si-DNMT1     –    –    +    +

E-cadherin

p53

DNMT1

β-Actin

**C**

si-Ctrl    si-p53
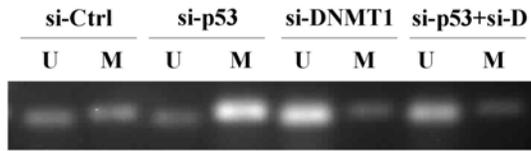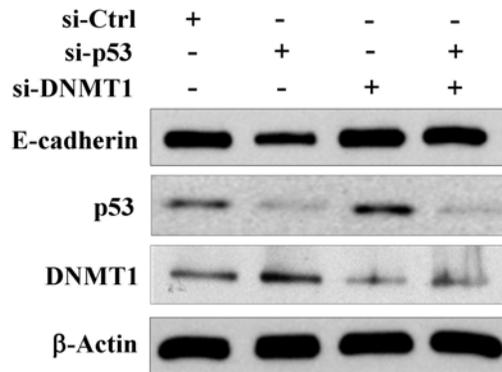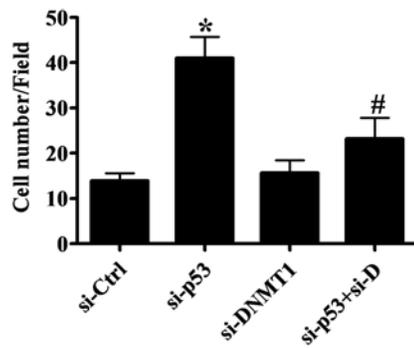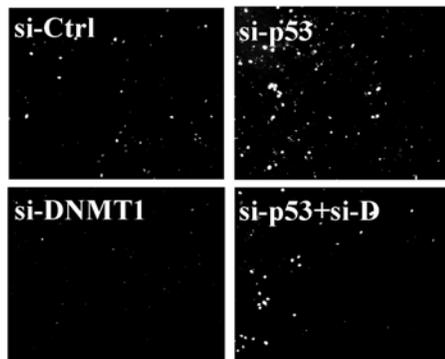
si-DNMT1    si-p53+si-D

**Figure 4. 8 DNMT1-mediated promoter methylation is involved in down-regulating E-cadherin when p53 is inhibited in SBOT3.1 cells**

**(A)** SBOT3.1 cells were transfected with non-targeting siRNA (si-Ctrl), p53 (si-p53) siRNA, DNMT1 (si-DNMT1) siRNA, or p53 plus DNMT1 siRNA for 72 hr. Methylation of the E-cadherin promoter was examined by methylation-specific PCR. Amplification was performed with primers specific for unmethylated (U) and methylated (M) DNA. **(B)** SBOT3.1 cells were transfected with non-targeting siRNA (si-Ctrl), p53 (si-p53) siRNA, DNMT1 (si-DNMT1) siRNA, or p53 plus DNMT1 (si-p53+si-D) siRNA for 72 hr. p53 and DNMT1 knockdowns and E-cadherin protein levels were examined by western blot. **(C)** SBOT3.1 cells were transfected with non-targeting siRNA (si-Ctrl), p53 (si-p53) siRNA, DNMT1 (si-DNMT1) siRNA, or p53 plus DNMT1 siRNA for 48 hr. Invasiveness was examined using the Matrigel-coated transwell assay. The upper panel shows representative fluorescent images from the invasion assay; the lower panel shows the summarized statistical results. Results are expressed as the mean ± SEM of three independent experiments. *$p < 0.05$ when compared to si-Ctrl. [#]$p < 0.05$ when compared to si-p53.
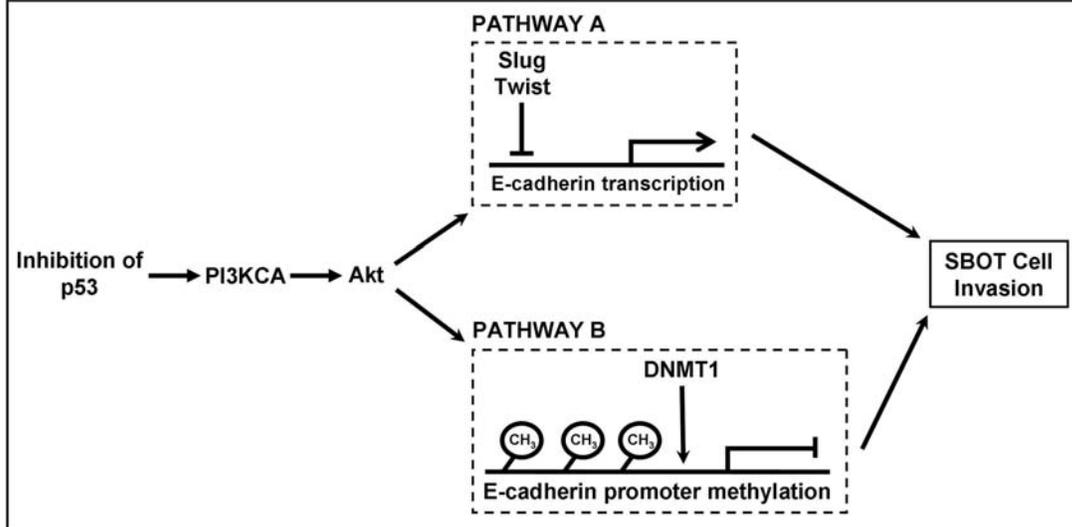
**Figure 4. 9 Proposed model for p53-regulated SBOT cell invasion**

Upon p53 loss during the progression from noninvasive SBOT to the invasive phenotype, the *PIK3CA* level is increased and subsequently enhances activation of Akt. The increased PI3K/Akt signaling leads to E-cadherin down-regulation by two pathways: A, it can increase the E-cadherin transcriptional repressors, Slug and Twist, which downregulate E-cadherin expression by suppressing transcription; B, it can increase DNA methyltransferase 1 (DNMT1) expression, which downregulates E-cadherin expression by increasing its promoter methylation. Once the expression of E-cadherin has been downregulated, SBOT cells become invasive.

# Chapter 5. EGF-induced EMT and invasiveness in serous borderline ovarian tumor cells: a possible step in the transition to low-grade serous carcinoma cells

## 5.1 Introduction

The epithelial-mesenchymal transition (EMT) is a highly conserved biological process during which there are multiple biochemical changes. This process results in the conversion of polarized, immotile epithelial cells into mesenchymal cells with a motile phenotype. This important process was initially recognized during critical phases of embryonic development, and recently, it has been shown that EMT is involved in promoting cancer cell invasion and metastasis (108).

A defining feature of EMT is a reduction in E-cadherin levels and a concomitant induction of N-cadherin (212). Loss of E-cadherin expression is mainly due to an up-regulation of Snail, Slug, Twist, ZEB1 and other transcription factors that repress E-cadherin (166). There is increasing evidence indicating that EMT is stimulated by signals from the tumor microenvironment, including a variety of growth factors and cytokines. In addition, EMT has been shown to be regulated by a series of intracellular signaling networks, including ERK1/2, PI3K/Akt, Smads, RhoB and β-catenin (254).

Epithelial ovarian cancer is the fifth leading cause of cancer-related deaths among women in developed countries. Most deaths from ovarian cancer are due to metastases that are resistant to conventional therapies. The epithelial growth factor receptor (EGFR) family consists of four members, EGFR (HER1), ErbB2 (HER2), ErbB3 (HER3), and ErbB4 (HER4), and has been shown to play an important role in metastasis and tumorigenesis in many types of human cancers (169, 255). Amplifications and overexpression of the EGFR

family have been reported in high-grade ovarian cancer and are associated with more aggressive clinical behavior and a poor prognosis (256, 257). It has been shown that EGF can induce EMT in ovarian surface epithelium (OSE) and ovarian cancer cells, suggesting that EGF may be involved in ovarian cancer pathogenesis and metastasis (173, 174). Ovarian cancer cells with low E-cadherin expression are more invasive, and the absence of E-cadherin expression in ovarian cancers is predictive of poor survival (190, 192). Serous borderline ovarian tumors (SBOT) are non-invasive and are considered to be distinct entities that give rise to invasive low-grade serous carcinomas (LGSC), which have a relatively poor prognosis when compared to SBOT and are unrelated to high-grade serous carcinomas (200). Studies using clinical samples have shown that EGFR is expressed in borderline ovarian tumors (256, 258). Although the function of EGFR signaling in cultured ovarian cancer cells has been studied, its function in the borderline tumors and in LGSC is still unknown due to the lack of a suitable *in vitro* model. We recently established an *in vitro* culture system with human SBOT cells. Cultured SBOT cells grow slowly, are essentially non-invasive and exhibit limited motility. These characteristics resemble the cells' behavior *in vivo* (48). Our recent study showed that p53 regulates the transition of SBOT cells from non-invasive to invasive ovarian carcinomas by activating the PI3K/Akt pathway and decreasing the expression of E-cadherin, indicating that EMT is a critical process for the regulation of SBOT cell invasion (232, 259).

In this study, we report for the first time that the EGFR is expressed in two cultured SBOT cell lines, SBOT3.1 and SBOT4-LT, and in two LGSC-derived cell lines, MPSC1 and ILGC cells, and that EGF treatment induces cell migration and invasion in all cell lines. Interestingly, EGF only induces the cadherin switch in SBOT cells, which leads to SBOT cell migration and invasion. We also show that the underlying mechanisms involve the activation

of the ERK1/2 and PI3K/Akt pathways. The information derived from this study provides critical insight into the role of EGFR activation in the down-regulation of E-cadherin, which plays a key role in increasing SBOT cell migration and invasion.

## 5.2 Material and methods

### Cell culture

The SBOT3.1 (48), SV40 LT-infected SBOT (SBOT4-LT) (232) and SV40 LT/ST immortalized LGSC (ILGC) (198) cell lines were established in our laboratory. SBOT and ILGC cells were grown in a 1:1 (v/v) mixture of M199/MCDB105 medium (Sigma, Oakville, ON) supplemented with 10% fetal bovine serum (FBS, Hyclone Laboratories Inc., Logan, UT). The MPSC1 cell line, which was established from a LGSC (provided by Dr. Ie-Ming Shih, Department of Pathology, Johns Hopkins Medical Institutions, Baltimore, MD), was maintained in RPMI 1640 (Invitrogen, Burlington, ON) supplemented with 10% FBS (208). Cultures were maintained at 37°C in a humidified atmosphere of 5% $CO_2$ in air.

### Antibodies and reagents

Polyclonal anti-EGFR and anti-β-actin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal anti-E-cadherin and anti-N-cadherin antibodies were obtained from BD Biosciences (Mississauga, ON). Monoclonal anti-phospho-EGFR (Tyr1173), anti-phospho-ERK1/2 (Thr202/Tyr204) anti-ZEB1 and anti-HER2 antibodies and polyclonal anti-ERK1/2, anti-phospho-p38 MAPK (Thr180/Tyr182), anti-p38 MAPK, anti-phospho-Akt (Ser473) and anti-Akt antibodies were obtained from Cell Signaling Technology (Danvers, MA). Polyclonal anti-Snail and anti-Slug antibodies were obtained from Abgent (San Diego, CA). Horseradish peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were obtained from Bio-Rad Laboratories

(Hercules, CA). Horseradish peroxidase-conjugated donkey anti-goat IgG was obtained from Santa Cruz Biotechnology. Human epidermal growth factor (EGF), AG1478, SB203580 and LY294002 were obtained from Sigma. PD98059 was obtained from Calbiochem (San Diego, CA).

**Treatment methods**

In the migration and invasion assays, cells with 80% confluence or cells treated with siRNA were treated with EGF for 24 (migration) and 48 (invasion) hr, respectively. After EGF treatment, cells were trypsinized and seeded into transwell inserts. For the general EGF treatment experiments, cells were cultured until 80% confluent and treated with 50 ng/ml EGF. The effect of EGF on the mRNA levels of E-cadherin, N-cadherin, Snail, Slug, Twist and ZEB1 were examined after 24 hr EGF treatment. The effect of EGF on the protein levels of those molecules were examined after 48 hr EGF treatment. The levels of specific mRNA and protein were examined by RT-qPCR and western blot, respectively. To knockdown EGFR, the cells were cultured until 60% confluent and then transfected with ON-TARGET*plus* SMART*pool* EGFR (50 nM) siRNA (Dharmacon Research, Inc., Lafayette, CO) using Lipofectamine RNAiMAX (Invitrogen) for 48 hr. The siCONTROL NON-TARGETING*pool* siRNA (Dharmacon) was used as the transfection control.

**Western blot**

Cells were lysed in lysis buffer (Cell Signaling Technology), and protein concentrations were determined using a DC protein assay kit with BSA as the standard (Bio-Rad Laboratories). Equal amounts of protein were separated by SDS polyacrylamide gel electrophoresis and transferred to PVDF membranes. Following blocking with TBS

containing 5% non-fat dry milk for 1 hr, membranes were incubated overnight at 4°C with primary antibodies, followed by incubation with HRP-conjugated secondary antibody. Immunoreactive bands were detected with enhanced chemiluminescent substrate. Membranes were stripped with stripping buffer and reprobed with anti-β-actin as a loading control.

**Reverse transcription quantitative real-time PCR**

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription was performed with 3 μg of RNA, random primers and M-MLV reverse transcriptase (Promega, Madison, WI). The primers used for SYBR Green reverse transcription-qPCR (RT-qPCR) were as follows: E-cadherin, 5'-ACA GCC CCG CCT TAT GAT T-3' (sense) and 5'-TCG GAA CCG CTT CCT TCA-3' (antisense); N-cadherin, 5'-GGA CAG TTC CTG AGG GAT CA-3' (sense) and 5'-GGA TTG CCT TCC ATG TCT GT-3' (antisense); Snail, 5'-CCC CAA TCG GAA GCC TAA CT-3' (sense) and 5'-GCT GGA AGG TAA ACT CTG GAT TAG A-3' (antisense); Slug, 5'-TTC GGA CCC ACA CAT TAC CT-3' (sense) and 5'-GCA GTG AGG GCA AGA AAA AG-3' (antisense); Twist, 5'-GGA GTC CGC AGT CTT ACG AG-3' (sense) and 5'-TCT GGA GGA CCT GGT AGA GG-3' (antisense); ZEB1, 5'- GCA CCT GAA GAG GAC CAG AG-3' (sense) and 5'-TGC ATC TGG TGT TCC ATT TT-3' (antisense); and GAPDH, 5'-GAG TCA ACG GAT TTG GTC GT-3' (sense) and 5'-GAC AAG CTT CCC GTT CTC AG-3' (antisense). RT-qPCR was performed on an Applied Biosystems 7300 Real-Time PCR System (Perkin-Elmer, Wellesley, MA) equipped with a 96-well optical reaction plate. All RT-qPCR experiments were run in triplicate, and a mean value was used for the determination of mRNA levels. Relative quantification of the mRNA levels was performed using the comparative Ct method with GAPDH as the reference gene and with the formula $2^{-\Delta\Delta Ct}$.

**Transwell migration and invasion assay**

Migration and invasion assays were performed in Boyden chambers with minor modifications (209). Cell culture inserts (24-well, pore size 8 μm; BD Biosciences, Mississauga, ON) were seeded with $1 \times 10^5$ cells in 250 μL of medium with 0.1% FBS. Un-coated inserts were used for migration assays, whereas inserts pre-coated with growth-factor-reduced Matrigel (40 μl, 1 mg/ml, BD Biosciences) were used for invasion assays. Medium with 10% FBS (750 μl) was added to the lower chamber and served as a chemotactic agent. After 24 hr (migration) or 48 hr (invasion) incubation, non-migrating/invading cells were wiped from the upper side of the membrane. Cells that penetrated the membrane were fixed with cold methanol, and cell nuclei were stained with Hoechst 33258 and counted by epifluorescence microscopy with Northern Eclipse 6.0 software (Empix Imaging, Mississauga, ON). Triplicate inserts were used for each individual experiment, and five microscopic fields were counted per insert.

**Statistical analysis**

Results are presented as the mean ± SEM of at least three independent experiments. Two-sample data were analyzed by Excel with the two-sample *t*-test assuming unequal variances. Multiple comparisons were analyzed by one-way ANOVA followed by Tukey's multiple comparison test using PRISM software. Significant differences were defined as $p < 0.05$.

## 5.3 Results

**Expression of E-cadherin, N-cadherin, EGFR and HER2 in cultured SBOT and LGSC cells**

Our recent studies showed that EMT is a critical process that contributes to the progression of non-invasive SBOT to invasive LGSC (232, 259). To confirm this result, we compared the basal expression levels of E-cadherin and N-cadherin in two SBOT lines, SBOT3.1 and SBOT4-LT, and two LGSC-derived cell lines, MPSC1 and ILGC. SBOT3.1 cells grew slowly, whereas SBOT4-LT, MPSC1 and ILGC cells were grew faster. As shown in Figure 5.1A, SBOT3.1 and MPSC1 exhibited an epithelial morphology. With the introduction of SV40 LT or LT/ST, SBOT4-LT and ILGC exhibited a more atypical and scattered morphology. To compare the expression levels of E-cadherin and N-cadherin, cells were grown until they were fully confluent, and then the total proteins were collected. As shown in Figure 5.1B, the expression levels of E-cadherin were high in SBOT3.1 cells and low in MPSC1 cells, whereas the levels of E-cadherin were almost absent in SV40 immortalized SBOT4-LT and ILGC cells, which is consistent with our previous data showing that E-cadherin is down-regulated by the inhibition of p53 (198, 232). These results indicate that MPSC1 cells are a more mesenchymal-like cell type compared to SBOT3.1 cells. To date, whether cultured SBOT and LGSC cells express EGFR or HER2 remains unclear. As shown in Figure 5.1C, both SBOT and LGSC cells expressed EGFR and HER2. The expression level of EGFR was lower in SBOT3.1 cells than in others, whereas all cell lines expressed similar levels of HER2.


**EGF treatment increases cell migration and invasion in SBOT and LGSC cells**

Transwell migration and invasion assays showed that SBOT3.1 cells were essentially

non-motile and non-invasive, whereas SBOT4-LT, MPSC1 and ILGC cells were highly motile and invasive (Figure 5.2A). Interestingly, EGF treatment resulted in a significant increase in cell migration (Figure 5.2B) and invasion (Figure 5.2C) in a dose-dependent manner in all SBOT and LGSC cell lines. To confirm the involvement of EGFR in EGF-induced cell invasion, EGFR-specific siRNA was used to knock down the endogenous EGFR. Western blot analysis showed that EGFR siRNA significantly knocked down the endogenous expression of EGFR. Moreover, EGFR siRNA abolished EGF-induced cell migration and invasion (Figure 5.2D). These results confirmed that EGFR is required for EGF-induced cell migration and invasion.

**EGF induces a down-regulation of E-cadherin and an up-regulation of N-cadherin in SBOT cells**

A characteristic of EMT is a switch from E-cadherin to N-cadherin expression. In SBOT3.1 and SBOT4-LT cells, RT-qPCR analysis showed that EGF treatment down-regulated E-cadherin mRNA levels. Concurrently, N-cadherin mRNA levels increased with EGF treatment. Unexpectedly, EGF treatment did not alter the mRNA levels of E-cadherin or N-cadherin in MPSC1 and ILGC cells (Figure 5.3A). Similarly, western blot analysis performed on total cell lysates collected from cells treated with EGF for 48 hr showed that EGF down-regulated E-cadherin and up-regulated N-cadherin total protein levels in SBOT3.1 cells, but not in MPSC1 cells (Figure 5.3B). In addition, the effects of EGF on the mRNA and protein levels of E- and N-cadherin in SBOT3.1 cells were eliminated by treatment with the EGFR inhibitor, AG1478 (Figures 5.3C and D). Moreover, EGFR siRNA abolished the EGF-induced switch from E-cadherin to N-cadherin (Figure 5.3E). It has been shown that the binding of EGF to EGFR rapidly induces clustering and

internalization of the ligand-receptor complexes, ultimately resulting in lysosomal degradation of both EGF and its receptor (260). This process was supported by the data in Figure 5.3E, which shows that EGFR was down-regulated in SBOT3.1 cells in response to EGF treatment.

**EGF up-regulates Snail, Slug and ZEB1, but not Twist, in SBOT cells**

To investigate whether EGF down-regulates E-cadherin expression by modulating the transcriptional regulation of E-cadherin, we used RT-qPCR to examine the mRNA levels of the E-cadherin transcriptional repressors Snail, Slug, Twist and ZEB1. Treatment with EGF significantly increased Snail, Slug and ZEB1 mRNA levels in SBOT3.1 and SBOT4-LT cells. However, EGF treatment did not alter the mRNA levels of Twist. In addition, the effects of EGF on these transcription factors were not detected in MPSC1 and ILGC cells, confirming that the E-cadherin is not transcriptionally regulated by EGF in LGSC cells (Figure 5.4A). In addition, treatment with AG1478 abolished the effects of EGF on Snail, Slug and ZEB1 mRNA levels in SBOT3.1 cells (Figure 5.4B). Moreover, western blot analysis showed that EGFR siRNA abolished EGF-induced Snail, Slug and ZEB1 expression in SBOT3.1 cells (Figure 5.4C).

**Activation of ERK1/2 and PI3K/Akt pathways are mediated by EGF-induced EMT and cell migration and invasion in SBOT cells**

It has been shown that the ERK1/2, p38 MAPK and PI3K/Akt pathways are involved in EGF-induced EMT (173, 197). However, it is unknown whether these signaling pathways are also involved in EGF-induced EMT in SBOT cells. As shown in Figure 5.5, treatment with EGF induced the activation of ERK1/2 and Akt with the maximal effect observed at 5 min

followed by a decrease after 180 min treatment. Interestingly, treatment with EGF did not activate p38 MAPK in SBOT3.1 cells. In contrast, EGF induced ERK1/2, p38 MAPK and Akt activation in MPSC1 cells. In SBOT3.1 cells, the EGF-induced down-regulation of E-cadherin and the up-regulation of N-cadherin mRNA and protein levels were diminished by treatment with the MEK1 inhibitor PD98059. Interestingly, treatment with the PI3K inhibitor LY294002 only diminished the EGF-induced down-regulation of E-cadherin but did not affect the EGF-induced up-regulation of N-cadherin (Figure 5.6A). In addition, treatment with PD98059 and LY294002 diminished EGF-induced up-regulation of Snail and Slug mRNA levels. However, the EGF-induced up-regulation of ZEB1 mRNA levels was only blocked by treatment with PD98059 and not with LY294002 (Figure 5.6B). Furthermore, EGF-induced cell migration and invasion were blocked by PD98059 and LY294002 treatments, although the inhibitory effect of LY294002 was less than that of PD98059 (Figure 5.6C). In MPSC1 cell, inhibition of ERK1/2, p38 MPAK and PI3K/Akt by PD98059, SB203580 and LY294002 attenuated EGF-induced cell migration and invasion (Figure 5.6D). Taken together, these results indicated that the ERK1/2 and PI3K/Akt pathways are involved in EGF-induced EMT and cell migration and invasion in SBOT cells. In addition, although EGF did not induce EMT in MPSC1 cells, our results indicate that ERK1/2, p38 MPAK and PI3K/Akt signaling pathways are involved in EGF-induced MPSC1 cell migration and invasion.

## 5.4 Discussion

There is increasing evidence indicating that the activation of EGFR signaling contributes to cellular invasion in ovarian cancer by a variety of mechanisms. EGF treatment is known to increase cultured ovarian cancer cell migration, invasion, and proteolytic activity (261, 262). Although the contributions of EGF and EGFR signaling have been described in

ovarian cancer, the majority of studies have been performed only on high-grade ovarian cancer cells. In borderline tumors, immunohistochemical studies have shown that EGF and the EGFR are expressed, but there is no difference in EGFR staining intensity between benign, borderline and malignant ovarian tumors (263, 264). Despite reports of EGFR expression in borderline tumors, the EGFR-mediated cell functions remain largely unknown. In the present study, we show that, consistent with previous immunohistochemical results, EGFR is expressed in cultured SBOT and LGSC cells. It is well known that SV40 large T antigen (LT) inactivates p53 and retinoblastoma protein (Rb), whereas SV40 small T antigen (ST) inhibits the activity of the protein phosphatase 2A (PP2A) (70, 265). It has been shown that the cell motility can be regulated by p53 and PP2A (265, 266). In the present study, we used two SBOT cell lines which one is infected with SV40 LT (SBOT4-LT) and the other one is not (SBOT3.1). In addition, ILGC is the SV40 LT/ST immortalized LGSC cell line, whereas MPSC1 is the LGSC-derived cell line which does not carry SV40 LT/ST. Interestingly, although the four cell lines used in this study have different genetic backgrounds, our results show that treatment with EGF induced cell migration and invasion in all SBOT and LGSC cell lines. These results suggest that p53/Rb and PP2A may not affect the EGF-induced cell migration and invasion in SBOT and LGSC cells.

It has been shown that none of the EGF family of peptides can bind HER2, and this is important because HER2 is the preferred dimerization partner for all the other EGFR family members (255). Overexpression of HER2 has been shown in high-grade ovarian cancer (267, 268). However, other studies showed no relationship between HER2 expression and survival among patients with high-grade ovarian cancer (269, 270). In SBOT and LGSC, similar to high-grade ovarian cancer, HER2 expression and its association with prognosis are controversial (271, 272). In the present study, we found that the expression levels of HER2

were similar in two SBOT and two LGSC cell lines. However, whether HER2 is involved in EGF-induced SBOT and LGSC cell motility remains unknown.

In ovarian cancer, based on molecular genetic and morphological studies, it has been suggested that there are two pathways of tumorigenesis that correspond to the development of low-grade and high-grade serous ovarian carcinoma (22). In type I tumors, invasive LGSC develops from a non-invasive SBOT. Histopathologic and molecular genetic studies suggest that SBOT may arise from ovarian surface epithelium (OSE) or cystadenomas (33). In humans, OSE has either a flat or a cuboidal appearance. Flat OSE does not express E-cadherin. In the ovary, E-cadherin expression is limited to rare regions such as cuboidal and columnar OSE, where cells resemble metaplastic epithelium (26, 273). Immunohistochemical studies showed that membranous E-cadherin expression is detected in benign and serous borderline ovarian tumors. Importantly, reduced expression of E-cadherin correlates with the presence of microinvasion in serous borderline tumors (191). Our recent study in cultured SBOT cells also showed that down-regulation of E-cadherin contributes to the progression of SBOT to invasive LGSC (232). Taken together, these results suggest that the expression of E-cadherin occurs intermittently during the progression from OSE to SBOT to invasive LGSC and may be required for the initiation of tumorigenesis in type I tumors. Therefore, we hypothesize that once normal OSE acquires the expression of E-cadherin, which may play a role in early events leading to the malignant phenotype, the subsequent EMT may be required for the progression of a non-invasive tumor to an invasive tumor.

Although the key feature of EMT is the down-regulation of E-cadherin and up-regulation of N-cadherin, there still are some other molecular markers that are used for EMT, such as increased expression of vimentin, fibronectin and nuclear localization of β-catenin and decreased expression of the tight junction protein, occluding (212). However,

the transition from epithelial to mesenchymal cell characteristics encompasses a spectrum of inter- and intracellular changes, not all of which are always seen during EMT (274). In the present study, we show that EGF treatment induced a switch from E-cadherin to N-cadherin expression in SBOT cells. However, the effect of EGF on other EMT markers requires further investigation. Here, we show that EGF treatment down-regulates E-cadherin expression in SBOT cells. In contrast, no such changes were observed in LGSC cells. The western blot results show that the EGFR level was higher in SBOT3.1 cells than in MPSC1 cells, indicating that the effects of EGF on cadherin switch are not related to the levels of EGFR. A recent study showed that different binding affinities between EGF and EGFR activate different signaling pathways. High-affinity EGF binding is sufficient for activation of most canonical signaling pathways, whereas low-affinity EGF binding is required for the activation of the STATs and PLCγ1 (275). Many signaling pathways have been reported to be involved in the EMT in ovarian cancer (276). It will require further investigation to examine whether the divergent effects of EGF on the cadherin switch result from the different binding affinities between EGF and EGFR in SBOT and LGSC cells. In high-grade ovarian cancer cells, we recently showed that $H_2O_2$ mediates the EGF-induced down-regulation of E-cadherin expression in SKOV3 ovarian cancer cells and suggested that the lack of an effect of EGF on E-cadherin in OVCAR3 cells may reflect an uncoupling of EGFR activation from $H_2O_2$ production (197). However, because the EGFR is functional, as shown by detection of activated EGF-induced EGFR phosphorylation, ERK1/2, p38 MAPK and PI3K/Akt, it is unclear whether the lack of an effect of EGF on E-cadherin expression in MPSC1 cells is due to the lack of $H_2O_2$ production after EGF treatment.

Reduced expression of E-cadherin in human cancers is associated with metastasis, whereas in high-grade ovarian cancer, forced expression of E-cadherin inhibits tumor

metastasis (277). We have shown that endogenous E-cadherin plays an important regulatory role in cell invasion and that EGF-induced cell invasion is mediated by the down-regulation of E-cadherin expression in high-grade ovarian cancer cells (197). In SBOT cells, our recent study showed that the down-regulation of E-cadherin by the PI3K/Akt pathway contributes to the progression to the invasive phenotype (232). In this study, we show that LGSC-derived MPSC1 cells express lower levels of E-cadherin and higher levels of N-cadherin than SBOT cells, suggesting that EMT may contribute to the progression from SBOT to invasive LGSC.

In the present study, our data demonstrate that in SBOT cells, ERK1/2 and Akt mediated the EGF-induced down-regulation of E-cadherin expression, whereas only ERK1/2 was involved in EGF-induced N-cadherin expression. Down-regulation of E-cadherin is mainly due to the up-regulation of Snail, Slug, Twist, ZEB1 and other transcription factors that repress E-cadherin (166). We show here that the expression of Snail, Slug and ZEB1, but not Twist, was increased by EGF treatment in SBOT cells. Recent studies have shown that Twist and ZEB1 not only repress E-cadherin expression but also induce the expression of N-cadherin (134, 278). Treatment with LY294002 did not block the EGF-induced up-regulation of N-cadherin, which may be due to the lack of an inhibitory effect of LY294002 on ZEB1 expression. Nevertheless, both the ERK1/2 and PI3K/Akt pathways were involved in EGF-induced SBOT cell migration and invasion. These results are consistent with our previous finding that E-cadherin, but not N-cadherin, plays an important role in the regulation of SBOT cell invasion (198, 232, 259).

In summary, this study demonstrates that EGFR is expressed in cultured SBOT and LGSC cells and that treatment with EGF induces cell migration and invasion by activating EMT in SBOT cells, which may play an important role in the progression from SBOT to invasive LGSC. In addition, this study suggests that there may be E-cadherin-independent

mechanisms that mediate the EGF-induced cell migration and invasion in LGSC cells.

**A**
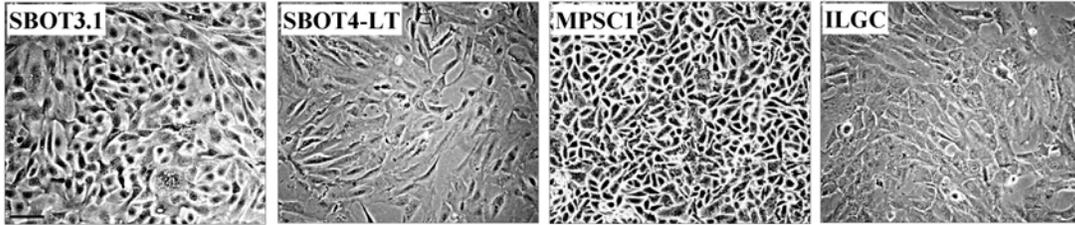


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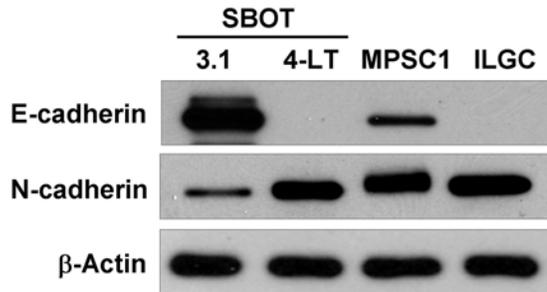


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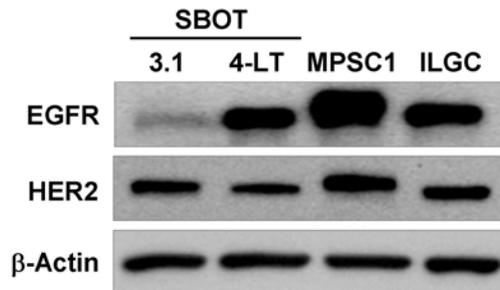


**Figure 5. 1 Expression of E-cadherin, N-cadherin, EGFR and HER2 in SBOT3.1, SBOT4-LT, MPSC1 and ILGC cells**

**(A)** The morphology of SBOT3.1, SBOT4-LT, MPSC1 and ILGC cells. The scale bar represents 100 μm. **(B)** Endogenous protein levels of E-cadherin and N-cadherin were analyzed by western blot. **(C)** Endogenous protein levels of EGFR and HER2 were analyzed by western blot.

**A**

SBOT3.1  SBOT4-LT    MPSC1  ILGC

Migration

Invasion

**B**

SBOT3.1

SBOT4-LT

MPSC1

ILGC

Cell migration (% of Ctrl)

EGF (ng/ml)

Ctrl  20  50  100

**C**

SBOT3.1

SBOT4-LT

MPSC1

ILGC

Cell invasion (% of Ctrl)

EGF (ng/ml)

Ctrl  20  50  100
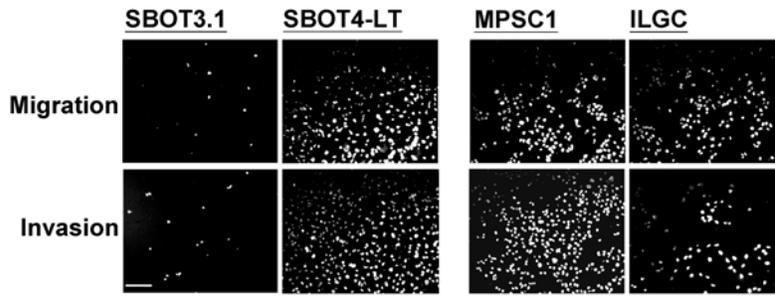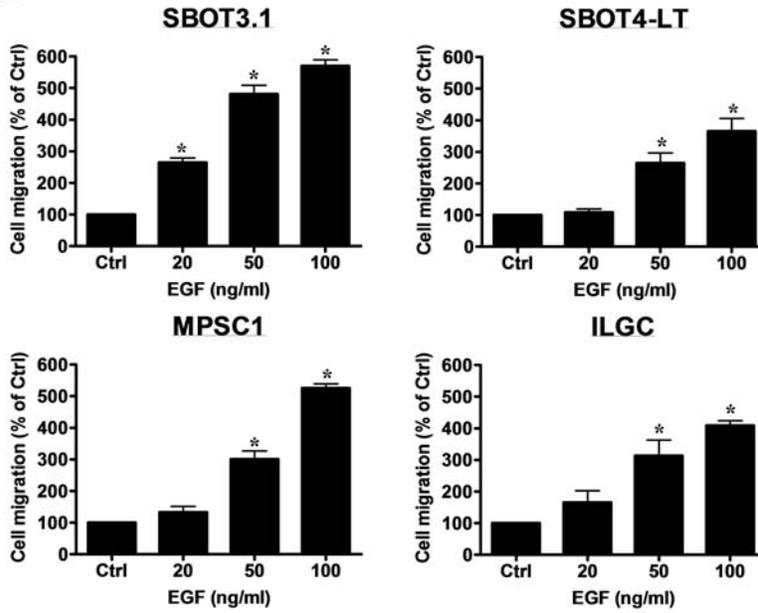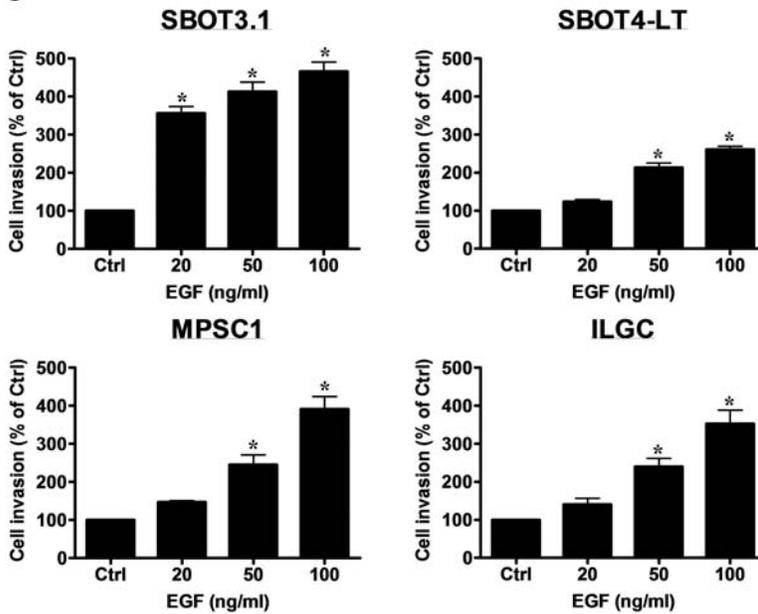
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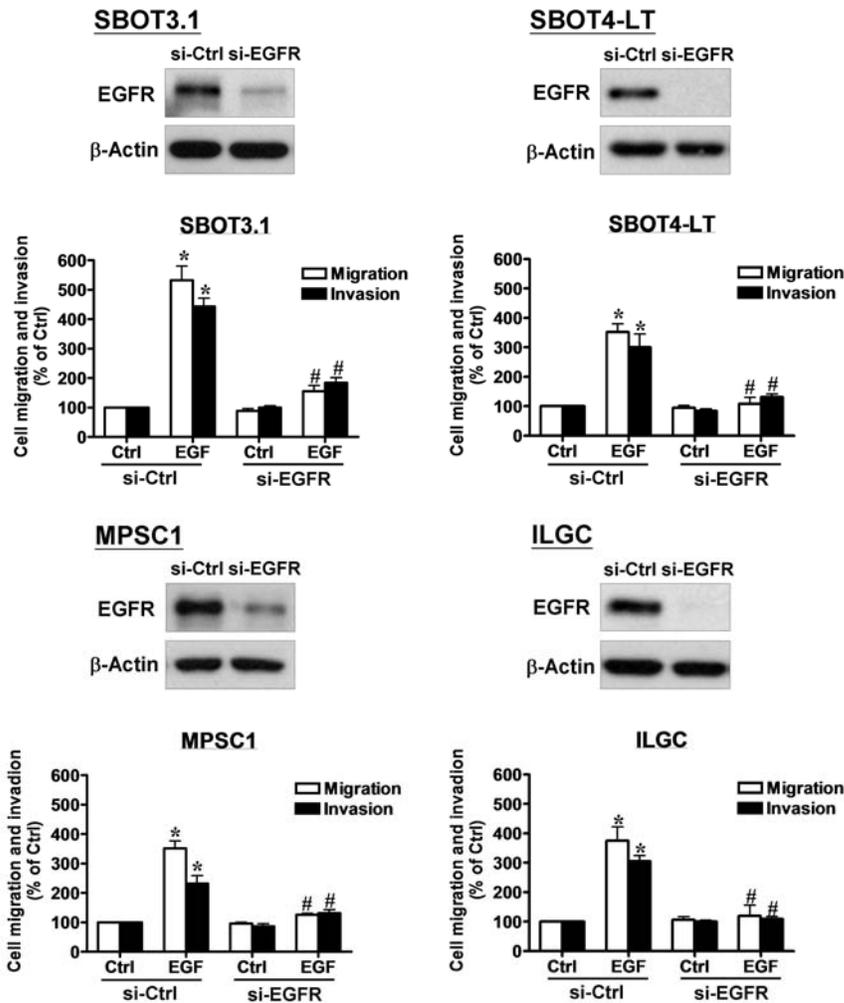


**Figure 5. 2 EGF induces cell migration and invasion in SBOT3.1, SBOT4-LT, MPSC1 and ILGC cells**

**(A)** The intrinsic migration and invasion of cells. **(B and C)** Cells were treated with increasing doses of EGF (20, 50 and 100 ng/ml). **(D)** Cells were transfected with control siRNA (si-Ctrl) or EGFR siRNA (si-EGFR) for 48 hr and then treated with 50 ng/ml EGF. After treatment cells were seeded into un-coated (migration) and Matrigel-coated (invasion) transwell inserts. After 24 hr (migration) and 48 hr (invasion) incubation, non-invading cells were wiped from the upper side of the filter and the nuclei of invading cells were stained with Hoechst 33258. Top panels show representative photos of the migration or invasion assay. Scale bar represents 200 μm. Bottom panels show summarized quantitative results which are expressed as the mean ± SEM of at least three independent experiments. Western blots show the knockdown of EGFR by EGFR siRNA. *p<0.05 compared with Ctrl. #p<0.05 compared with EGF or EGF in si-Ctrl.

**A**

## SBOT3.1

### E-cadherin



### N-cadherin



## SBOT4-LT

### E-cadherin



### N-cadherin



## MPSC1

### E-cadherin



### N-cadherin



## ILGC

### E-cadherin



### N-cadherin



110

**B**

**C**

<u>SBOT3.1</u>

**E-cadherin**



**N-cadherin**



**D**
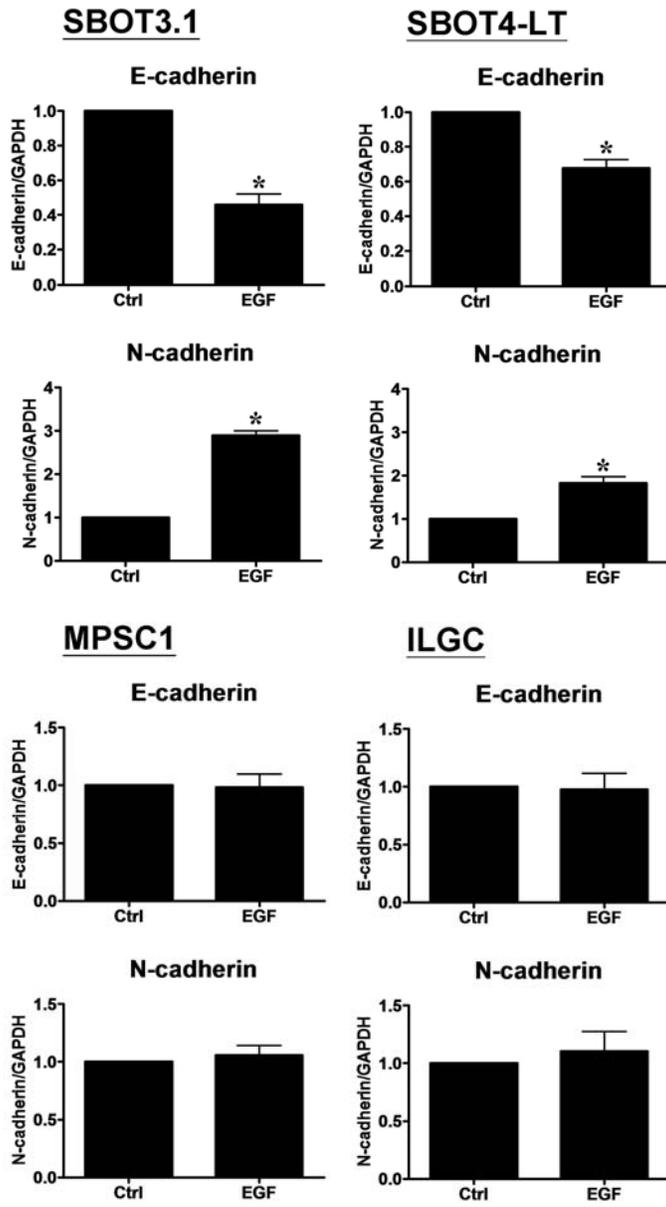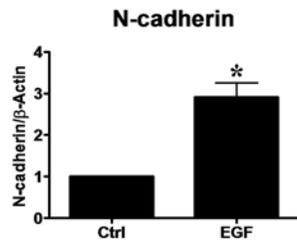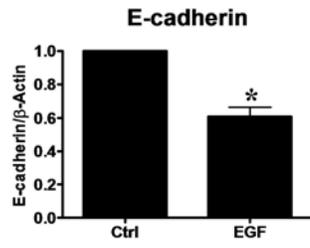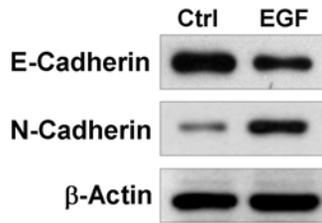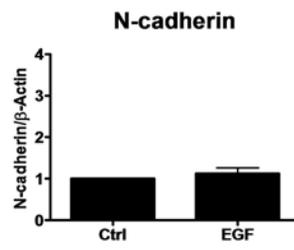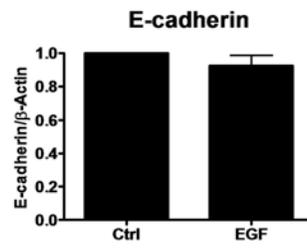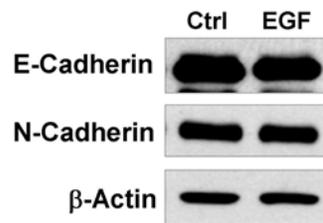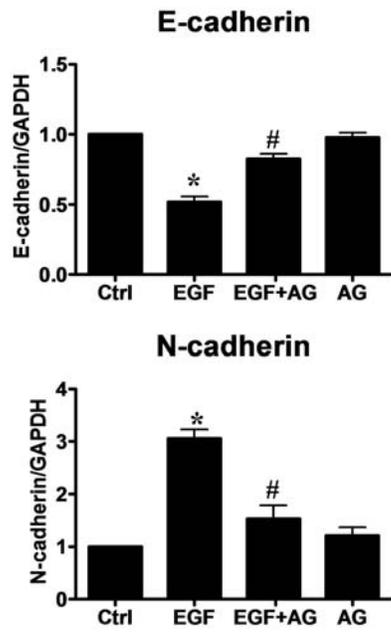
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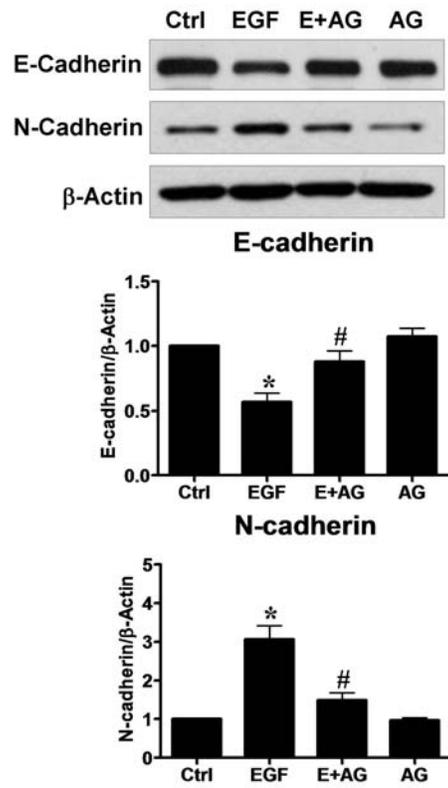


**E-cadherin**
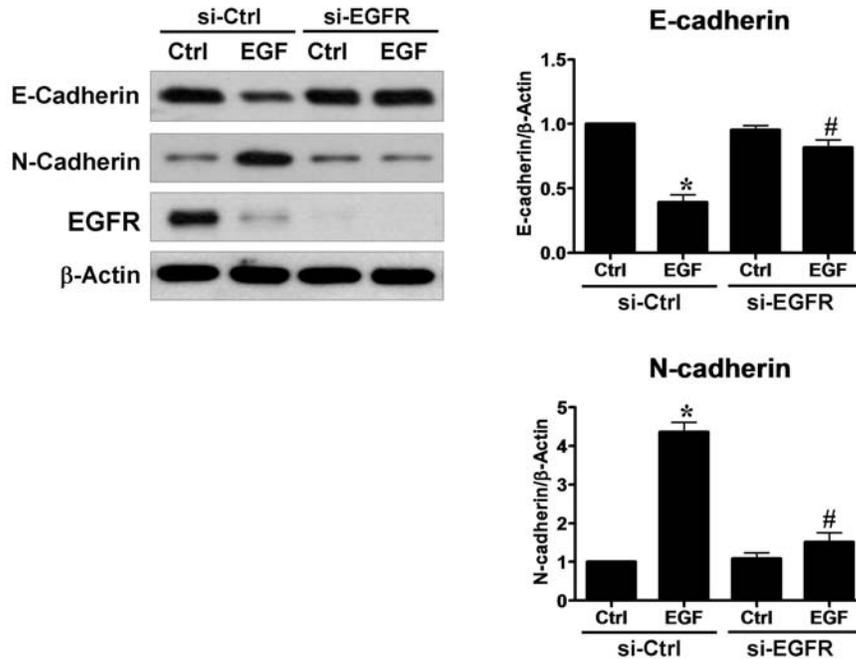


**N-cadherin**

**Figure 5. 3 EGF induces cadherin switch in SBOT3.1 and SBOT4-LT cells, but not in MPSC1 and ILGC cells**

**(A)** Cells were treated with 50 ng/ml EGF for 24 hr. E-cadherin and N-cadherin mRNA levels were analyzed by RT-qPCR. **(B)** Cells were treated with 50 ng/ml EGF for 48 hr. E-cadherin and N-cadherin protein levels were analyzed by western blot. **(C and D)** SBOT3.1 cells were treated with the EGFR inhibitor, AG1478 (10 μM), in the presence or absence of 50 ng/ml EGF, and the levels of E-cadherin and N-cadherin mRNA (24 hr EGF treatment) and protein (48 hr EGF treatment) were analyzed. E, SBOT3.1 cells were transfected with control siRNA (si-Ctrl) or EGFR siRNA (si-EGFR) for 48 hr and then treated with 50 ng/ml EGF for 48 hr. The protein levels of E-cadherin and N-cadherin were analyzed by western blot. The results are expressed as the mean ± SEM of at least three independent experiments. *p<0.05 compared with time-matched Ctrl. [#]p<0.05 compared with EGF or EGF in si-Ctrl.

**A**
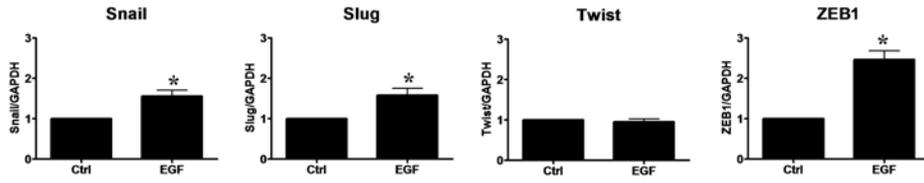
**SBOT3.1**



**SBOT4-LT**
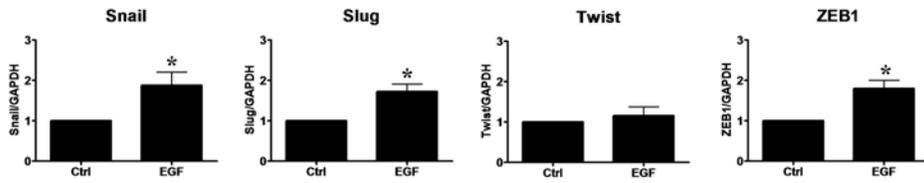


**MPSC1**
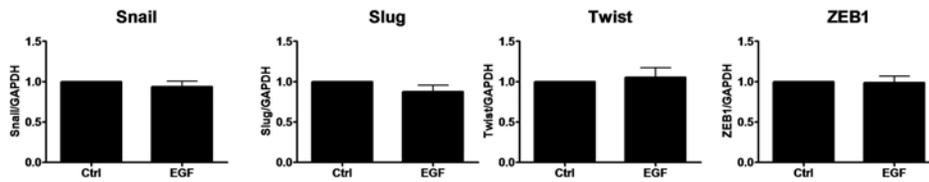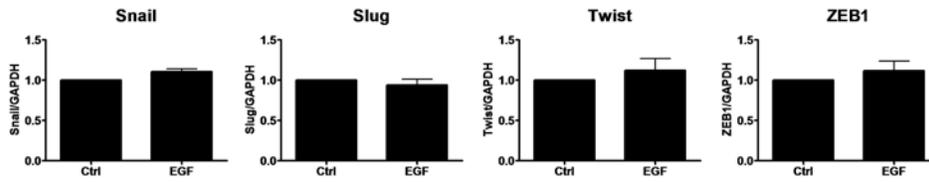


**ILGC**



**B**

**SBOT3.1**
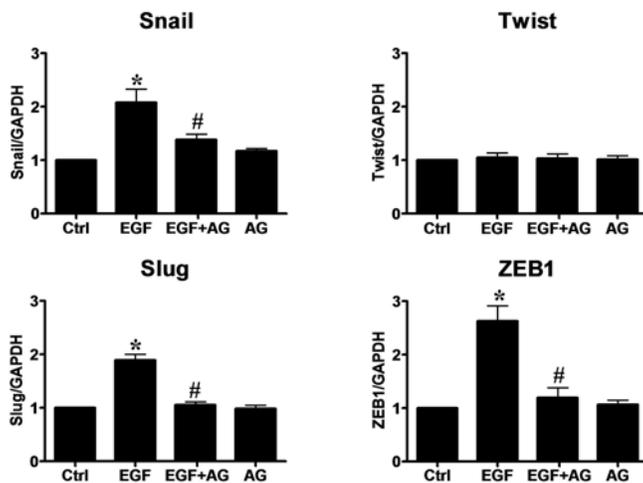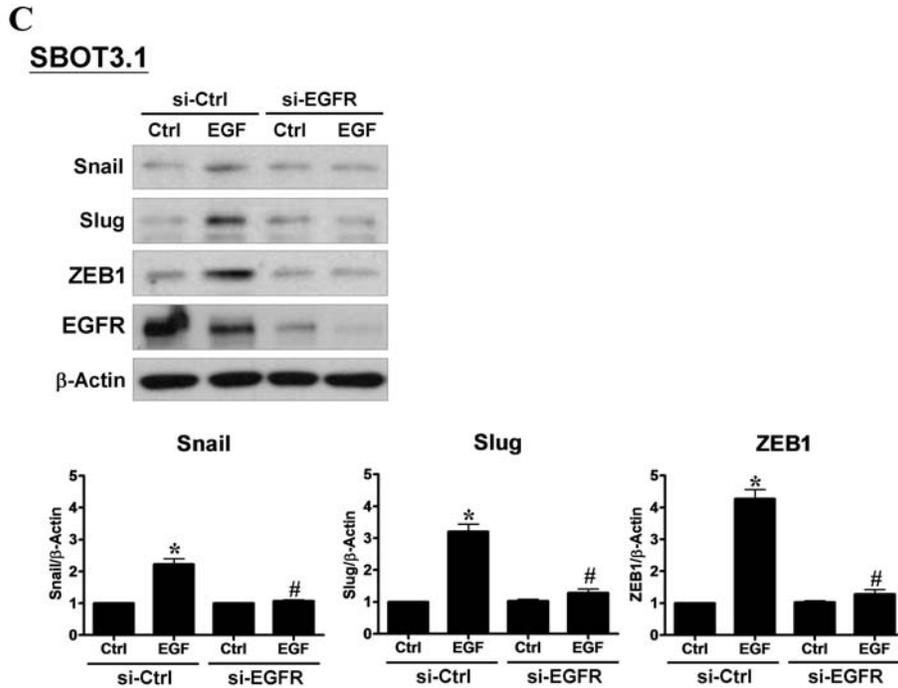
**Figure 5. 4 EGF induces Snail, Slug and ZEB1 expression in SBOT3.1 and SBOT4-LT cells, but not in MPSC1 and ILGC cells**

**(A)** Cells were treated with 50 ng/ml EGF for 24 hr, and the mRNA levels of Snail, Slug, Twist and ZEB1 were analyzed by RT-qPCR. **(B)** SBOT3.1 cells were treated with AG1478 (10 μM) in the presence or absence of 50 ng/ml EGF for 24 hr, and mRNA levels were analyzed by RT-qPCR. **(C)** SBOT3.1 cells were transfected with control siRNA (si-Ctrl) or EGFR siRNA (si-EGFR) for 48 hr and then treated with 50 ng/ml EGF for 48 hr. The protein levels of Snail, Slug and ZEB1 were analyzed by western blot. The results are expressed as the mean ± SEM of at least three independent experiments. *p<0.05 compared with Ctrl. #p<0.05 compared with EGF or EGF in si-Ctrl.
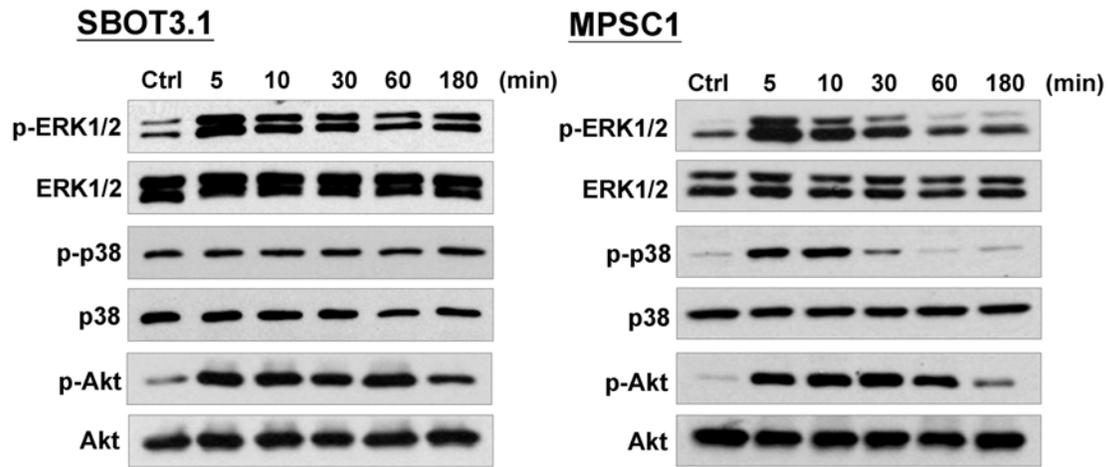
**Figure 5. 5 EGF activates ERK1/2 and Akt pathways in SBOT3.1 cells**

SBOT3.1 and MPSC1 cells were treated with 50 ng/ml EGF for the indicated durations. Phosphorylation of ERK1/2, p38 MAPK and Akt were determined by western blot using antibodies specific for phosphorylated, activated forms of ERK1/2 (p-ERK1/2), p38 MAPK (p-p38) and Akt (p-Akt). Membranes were stripped and reprobed with antibodies to total ERK1/2, p38 MAPK and Akt.

**A**

SBOT3.1
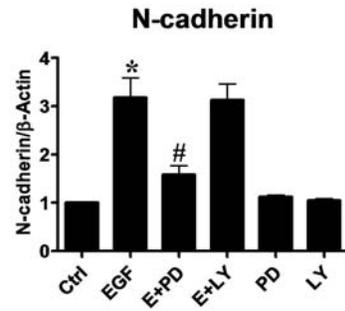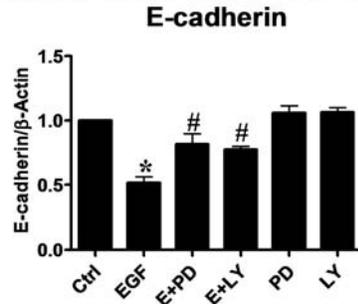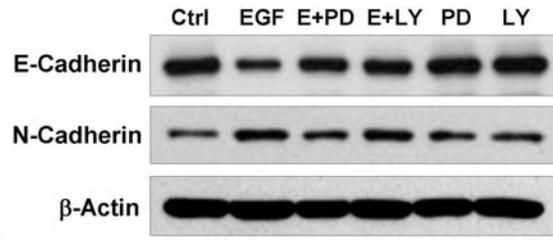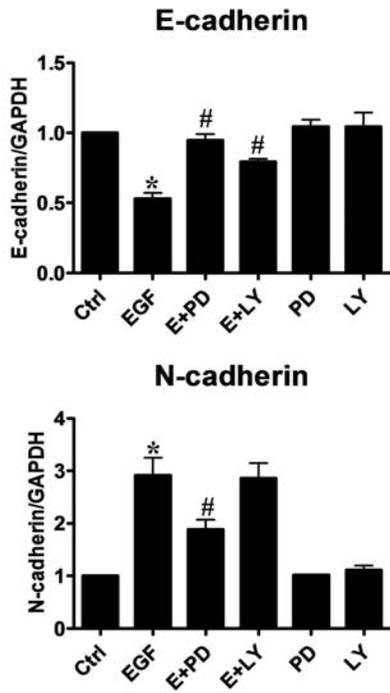
**B**

<u>SBOT3.1</u>
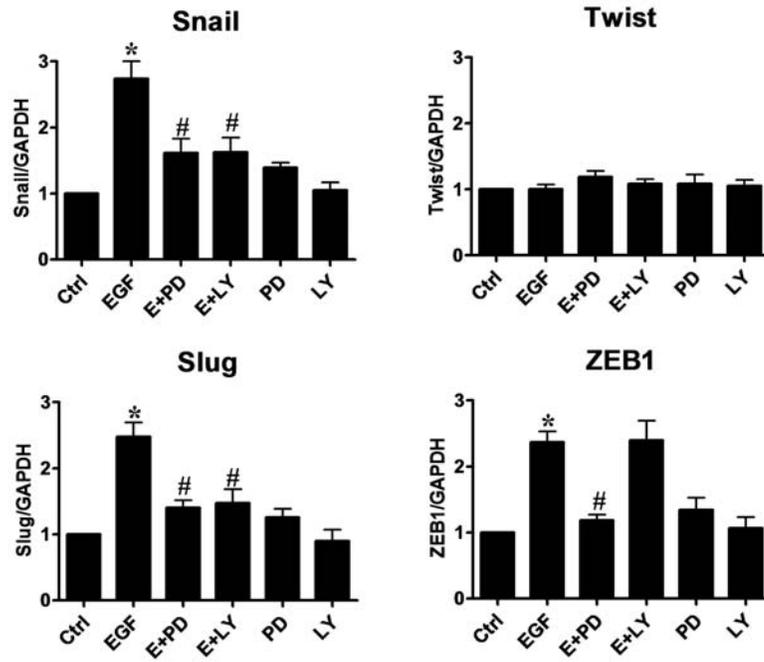


**C**
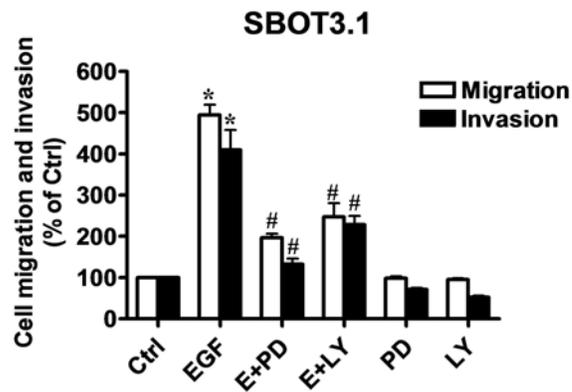
**D**
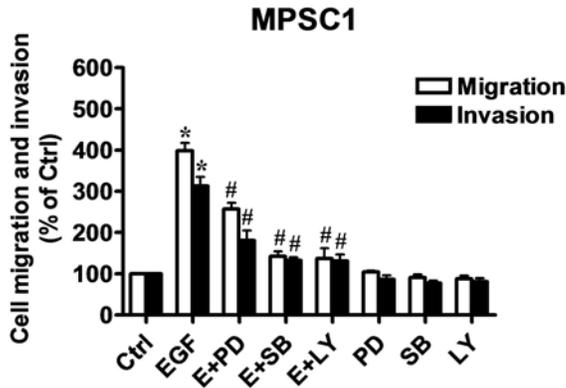


**Figure 5. 6 EGF induces cadherin switch through ERK1/2 and Akt activation in SBOT3.1 cells**

**(A)** SBOT3.1 cells were treated for 48 hr with PD98059 (20 μM) or LY294002 (20 μM) in the presence or absence of 50 ng/ml EGF. E-cadherin and N-cadherin mRNA (left panel) and protein (right panel) levels were analyzed by RT-qPCR and western blot, respectively. **(B)** SBOT3.1 cells were treated for with PD98059 (20 μM) or LY294002 (20 μM) in the presence or absence of 50 ng/ml EGF and Snail, and the Slug, Twist and ZEB1 mRNA levels were analyzed by RT-qPCR. **(C)** SBOT3.1 cells were treated with 50 ng/ml EGF in combination with PD98059 (20 μM) or LY294002 (20 μM). **(D)** MPSC1 cells were treated with 50 ng/ml EGF in combination with PD98059 (20 μM) SB203580 (10 μM) or LY294002 (20 μM). After treatment, cells were seeded into un-coated (migration) and Matrigel-coated (invasion) transwell inserts. After 24 hr (migration) and 48 hr (invasion) incubation, non-invading cells were wiped from the upper side of the filter and the nuclei of invading cells were stained with Hoechst 33258. Results are expressed as the mean ± SEM of at least three independent experiments. *p<0.05 compared with Ctrl. #p<0.05 compared with EGF.

# Chapter 6. TGF-beta induces serous borderline ovarian tumor cell invasion by activating EMT but triggers apoptosis in low-grade serous ovarian carcinoma cells

## 6.1 Introduction

Transforming growth factor-beta (TGF-β) is a pleiotropic cytokine that regulates cell proliferation, apoptosis, differentiation, migration and invasion (279). TGF-β signals through transmembrane type I (TβRI) and type II (TβRII) receptors to initiate downstream signaling (280). In the canonical pathway, TGF-β binding to TβRII recruits and phosphorylates TβRI, which results in TβRI activation. Activated TβRI phosphorylates the receptor-regulated Smad proteins Smad2 and Smad3. Phosphorylated Smad2 and Smad3 then co-associate with Smad4, translocate into the nucleus and regulate gene expression by binding to Smad-specific binding elements in the promoters of TGF-β-regulated genes (281). In humans, TGF-β overexpression has been detected in many cancer types and correlates with tumor metastasis, progression and prognosis (282, 283). Many studies have indicated that TGF-β can function as a tumor suppressor and promoter depending on the context (186). TGF-β acts as a tumor suppressor by inhibiting cell proliferation, while as a tumor promoter, TGF-β induces an epithelial-mesenchymal transition (EMT), cell motility and invasion (284).

EMT has been recognized as a key process for embryonic development and metastasis (160). Cells undergoing EMT down-regulate the expression of the E-cadherin epithelial marker and increase the expression of N-cadherin, a mesenchymal marker. This process has been shown proceed through a set of transcription factors including the Snail and Slug zinc-finger proteins, the Twist bHLH factor and the ZEB1 zinc-finger protein (285). TGF-β is a potent inducer of EMT, which was first recognized in cultured normal mammary

epithelial cells (176). TGF-β can induce EMT by activating Smad-dependent and Smad-independent pathways (177). Ectopic expression of Smad2 or Smad3 with Smad4 enhances EMT, whereas ectopic expression of dominant-negative Smad2, Smad3 or Smad4 blocks TGF-β-induced EMT (182).

TGF-β acts as a tumor suppressor in the early stages of cancer progression, and it becomes a tumor promoter in later stages (283). TGF-β1, TGF-β2 and TGF-β3 overexpression has been reported in human ovarian tumors (286). Ovarian cancer is thought to arise from normal ovarian surface epithelium (OSE) (26). TGF-β has been shown to inhibit human OSE proliferation and induce apoptosis, which may prevent the over-proliferation of cells during a normal ovulatory cycle (287). In the later stages of ovarian cancer, TGF-β enhances tumor cell proliferation and promotes metastasis by inducing an EMT (187, 288).

It has recently been recognized that high-grade serous ovarian carcinoma (HGSC) and low-grade serous ovarian carcinoma (LGSC) are fundamentally different types of tumors that develop from distinct molecular pathways (22). Compared with HGSC, LGSC accounts for a small proportion (9%) of all serous ovarian carcinomas (52). Invasive LGSC is developed from non-invasive borderline serous ovarian tumors (SBOT) (200, 289). In ovarian cancer, TGF-β-induced EMT is believed to play an important role in the regulation of cell invasion and metastasis (276). It has been shown that TGF-β and TβRII are expressed in primary human borderline ovarian tumors (286). Although the function of TGF-β in HGSC has been extensively investigated, to our knowledge, no study has examined the effects of TGF-β in the SBOT/LGSC system. Our recent studies demonstrate that E-cadherin down-regulation induces SBOT cell invasion, suggesting that EMT is involved in the progression from non-invasive SBOT to invasive LGSC (232, 290, 291). Thus, this study was undertaken to

test the hypothesis that TGF-β induces SBOT invasion by activating EMT.

## 6.2 Material and methods

**Cell culture**

The SBOT3.1 (48) and SV40 LT/ST immortalized LGSC (ILGC) (198) cell lines were established in our laboratory. SBOT and ILGC cells were grown in a 1:1 (v/v) mixture of M199/MCDB105 medium (Sigma, Oakville, ON) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories Inc., Logan, UT). The MPSC1 cell line, which was established from a LGSC (provided by Dr. Ie-Ming Shih, Department of Pathology, Johns Hopkins Medical Institutions, Baltimore, MD), was maintained in RPMI 1640 (Invitrogen, Burlington, ON) supplemented with 10% FBS (208). Cultures were maintained at 37°C in a humidified 5% $CO_2$ atmosphere in air.

**Antibodies and reagents**

The polyclonal anti-β-actin antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal anti-E-cadherin and anti-N-cadherin antibodies were obtained from BD Biosciences (Mississauga, ON). The monoclonal anti-phospho-Smad3, anti-Smad3, anti-Smad2, polyclonal anti-TGF-β receptor I, anti-TGF-β receptor II, anti-phospho-Smad2, anti-Smad4 and anti-caspase-3 antibodies were obtained from Cell Signaling Technology (Danvers, MA). Horseradish peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were obtained from Bio-Rad Laboratories (Hercules, CA). Horseradish peroxidase-conjugated donkey anti-goat IgG was obtained from Santa Cruz Biotechnology. Recombinant human TGF-β1 was obtained from R&D Systems (Minneapolis, MN). SB431542 was obtained from Sigma.

**Small interfering RNA (siRNA) transfection**

To knock down endogenous TGF-β receptor I (TβRI), cells were transfected with 50 nM ON-TARGET*plus* SMART*pool* TβRI siRNA (Dharmacon, Lafayette, CO) using Lipofectamine RNAiMAX (Invitrogen, Burlington, ON). The siCONTROL non-targeting siRNA pool (Dharmacon) was used as a transfection control. The knockdown efficiency was examined by RT-qPCR or western blot analysis.

**Western blot**

Equal protein amounts were separated by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes. The membranes were blocked with Tris-buffered saline containing 5% non-fat dry milk for 1 hr. The membranes were then incubated overnight at 4°C with primary antibodies followed by incubation with HRP-conjugated secondary antibodies. Immunoreactive bands were detected using an enhanced chemiluminescent substrate. The membranes were stripped with stripping buffer at 50ºC for 30 min and reprobed with anti-β-actin as a loading control.

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

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription was performed using 3 μg RNA, random primers and M-MLV reverse transcriptase (Promega, Madison, WI). The primers used for SYBR Green reverse transcription-qPCR (RT-qPCR) were as follows: TβRI: 5'-GTT AAG GCC AAA TAT CCC AAA CA-3' (sense) and 5'- ATA ATT TTA GCC ATT ACT CTC AAG G-3' (antisense); E-cadherin: 5'-ACA GCC CCG CCT TAT GAT T-3' (sense) and 5'-TCG GAA CCG CTT CCT TCA-3' (antisense); N-cadherin: 5'-GGA CAG TTC CTG AGG GAT

CA-3' (sense) and 5'-GGA TTG CCT TCC ATG TCT GT-3' (antisense); Snail: 5'-CCC CAA

TCG GAA GCC TAA CT-3' (sense) and 5'-GCT GGA AGG TAA ACT CTG GAT TAG A-3'

(antisense); Slug: 5'-TTC GGA CCC ACA CAT TAC CT-3' (sense) and 5'-GCA GTG AGG

GCA AGA AAA AG-3' (antisense); Twist: 5'-GGA GTC CGC AGT CTT ACG AG-3' (sense)

and 5'-TCT GGA GGA CCT GGT AGA GG-3' (antisense); ZEB1: 5'-GCA CCT GAA GAG

GAC CAG AG-3' (sense) and 5'-TGC ATC TGG TGT TCC ATT TT-3' (antisense); and

GAPDH: 5'-GAG TCA ACG GAT TTG GTC GT-3' (sense) and 5'-GAC AAG CTT CCC

GTT CTC AG-3' (antisense). RT-qPCR was performed using the Applied Biosystems 7300

Real-Time PCR System (Perkin-Elmer), which was equipped with a 96-well optical reaction

plate. All RT-qPCR experiments were performed in triplicate, and the mean value was used

for to determine the mRNA levels. Relative quantification of the mRNA levels was

performed using the comparative Ct method with GAPDH as the reference gene and using the

$2^{-\Delta\Delta Ct}$ formula.


**Transwell invasion assay**

Invasion assays were performed in Boyden chambers with minor modifications (209).

Cell culture inserts (24-well, 8 μm pore size; BD Biosciences, Mississauga, ON) were seeded

with $1\times10^5$ cells in 250 μl of medium supplemented with 0.1% FBS. Inserts pre-coated with

growth factor-reduced Matrigel (40 μl, 1 mg/ml, BD Biosciences) were used for the invasion

assays. Medium supplemented with 10% FBS (750 μl) was added to the lower chamber and

served as a chemotactic agent. After 48 hr incubation, non-invading cells were removed from

the upper side of the membrane. Cells that penetrated the membrane were fixed with cold

methanol, and cell nuclei were stained with Hoechst 33258 and counted by epifluorescence

microscopy using Northern Eclipse 6.0 software (Empix Imaging, Mississauga, ON).

Triplicate inserts were used for each individual experiment.

**Statistical analysis**

Results are presented as the mean ± SEM of at least three independent experiments and were analyzed by the one-way ANOVA test followed by Tukey's test using PRISM software. A $p<0.05$ was considered statistically significant.

## 6.3 Results

**TGF-β induces SBOT3.1 cell invasion but decreases the invasion of MPSC1 cells**

To test the hypothesis that TGF-β affects SBOT and LGSC cell invasion by activating EMT, we used SBOT3.1 and a LGSC-derived cell line MPSC1. Western blot analysis demonstrated that TGF-β type I (TβRI) and type II (TβRII) were expressed in SBOT3.1 and MPSC1 cells (Figure 6.1A). The TβRI expression level was higher in MPSC1 cells than SBOT3.1 cells although no significant difference was observed for the TβRII expression levels (Figure 6.1A). To examine the effect of TGF-β on cell invasion, Matrigel-coated transwells were used. As shown in Figure 1B, SBOT3.1 cells were non-invasive, whereas MPSC1 cells were highly invasive. Treatment with TGF-β (1, 10 and 20 ng/ml) significantly induced SBOT cell invasion. Surprisingly, TGF-β treatment significantly decreased the invasiveness of MPSC1 cells. The maximal effect of TGF-β was observed at 10 ng/ml in both cases (Figure 6.1B).

**TGF-β induces apoptosis in MPSC1, but not in SBOT3.1 cells**

To investigate the possible mechanism that mediated the inhibitory effect of TGF-β on MPSC1 cell invasion, we examined the TGF-β effect on apoptosis in SBOT3.1 and MPSC1 cells. TGF-β treatment for 48 hr induced EMT-like morphological changes in SBOT3.1 cells

from a cobblestone-like morphology to a fibroblastic-spindle shape. In contrast, the number of cells was lower in TGF-β-treated MPSC1 cells, suggesting that TGF-β had a pro-apoptotic effect (Figure 6.2A). To further confirm this result, the cleaved caspase-3 levels were examined after TGF-β treatment. As shown in Figure 6.2B, TGF-β treatment for 24 hr did not affect the expression of cleaved caspase-3 in SBOT3.1 cells. However, TGF-β induced cleaved caspase-3 expression in MPSC1 cells. Furthermore, the pro-apoptotic effect of TGF-β on SBOT3.1 and MPSC1 cells was examined using a trypan blue exclusion assay. Similar to the results obtained from the cleaved caspase-3 assay, TGF-β treatment for 48 hr decreased the number of MPSC1 but not SBOT3.1 cells (Figure 6.2C). These TGF-β effects on apoptosis were in agreement with the invasion assay results.

**TGF-β induces phosphorylation of Smad3, but not Smad2, in SBOT3.1 and MPSC1 cells**

The Smad signaling pathway is important for regulating numerous TGF-β-mediated cellular functions. Western blot analysis demonstrated that the co-Smad Smad4 was expressed in both cell lines, although the level was higher in MPSC1 cells (Figure 6.3A). TGF-β treatment induced Smad3 phosphorylation in a time-dependent manner in SBOT3.1 and MPSC1 cells. However, in both cell lines, TGF-β did not alter the Smad2 phosphorylation level (Figure 6.3B). SB431542 is a potent and specific TβRI inhibitor (292). Treatment with SB431542 significantly abolished the TGF-β-induced phosphorylation of Smad3 (Figure 6.3C) and the TGF-β-induced change in the cell invasion capability of SBOT3.1 and MPSC1 cells (Figure 6.4A). Moreover, the TGF-β effects on cell invasion were abolished by siRNA-mediated depletion of the TβRI receptor (Figure 6.4B). TGF-β-induced caspase-3 cleavage was eliminated by co-treatment with SB431542 and

TβRI depletion using siRNA (Figure 6.4C). To further confirm the apoptotic effect of TGF-β in LGSC cells, SV40 LT/ST immortalized LGSC (ILGC) cells were used. As shown in Figure 6.4D, TGF-β treatment increased cleaved caspase-3 expression, which was abolished by SB431542 co-treatment and TβRI depletion by siRNA. In addition, TGF-β-decreased the MPSC1 and ILGC cell numbers were abolished by TβRI depletion by siRNA (Figure 6.4E).

**TGF-β induces EMT by up-regulating Snail, Slug, Twist and ZEB1 in SBOT3.1 cells**

We next sought to better understand the mechanisms that mediate the TGF-β-induced EMT in SBOT3.1 cells. A switch from E- to N-cadherin expression has been suggested to be a key feature during EMT. RT-qPCR analysis demonstrated that TGF-β treatment down-regulated E-cadherin mRNA levels in SBOT3.1 cells while the N-cadherin mRNA levels increased (Figure 6.5A). These effects were confirmed at the protein level by western blot analysis following TGF-β treatment for 24 and 48 hr; SBOT3.1 cells demonstrated E-cadherin down-regulation and N-cadherin up-regulation at the total protein levels (Figure 6.5B). To investigate whether TGF-β down-regulates E-cadherin expression by modulating E-cadherin transcriptional regulation, we used RT-qPCR to examine the mRNA levels of the E-cadherin transcriptional repressors Snail, Slug, Twist and ZEB1. TGF-β treatment significantly increased the Snail, Slug, Twist and ZEB1 mRNA levels in SBOT3.1 cells (Figure 6.5C). SB431542 treatment and TβRI depletion by siRNA abolished the TGF-β effects on E- and N-cadherin mRNA and protein levels (Figures 6.6A and B). In addition, the TGF-β-induced changes to the Snail, Slug, Twist and ZEB1 mRNA levels were abolished by co-treatment with SB431542 and TβRI depletion by siRNA (Figures 6.6C and D).

## 6.4 Discussion

SBOT and LGSC have been recognized as entities that are distinct from HGSC (293). In this study, we used a cell culture system to demonstrate for the first time that TGF-β receptors are expressed in SBOT3.1 and MPSC1 cells. Interestingly, we also found that TGF-β exhibited a dual function whereby it induced SBOT3.1 cell invasion by activating an EMT, and it promoted apoptosis in MPSC1 cells.

Increasing evidence indicate that TGF-β functions as a tumor suppressor in early stage tumors while paradoxically acting as a tumor promoter in advanced cancers (186, 294). The molecular nature of this switch is complicated, perhaps context dependent and remains largely unknown. In contrast to HGSC, which presents as a clinically aggressive neoplasm that grows, rapidly spreads and is associated with poor outcome, LGSC maintains its low-grade appearance and low proliferative index (22, 53) which may explain the TGF-β apoptotic effects on LGSC cells. However, the role of this dual TGF-β function in the progression of SBOT to LGSC requires further investigation. It has been recognized that the expression level of other endogenous factors present in tumor cells may affect the tumor cell autonomous switch of the TGF-β response from tumor suppressor to promoter (186). LGSC develop in a stepwise manner from OSE and SBOT (22). p53 mutations are rarely detected in SBOT and LGSC (22). We have previously shown that p53 is wild-type in SBOT3.1 cells (232). Microarray analysis of tumor specimens demonstrates that the p53 level is increased in SBOT compared to OSE, but the p53 level is decreased when SBOT progresses to LGSC (54). Our western blot results confirmed that the p53 level is higher in SBOT3.1 than MPSC1 (Figure 6.7). We have shown that TGF-β induces cell growth arrest in normal human OSE and SV40 large T immortalized human OSE (IOSE) (287). Together with the current study, our results indicate that TGF-β functions as a tumor suppressor in OSE, IOSE, MPSC1 and

ILGC, which all have low levels of wild-type p53 or inactive p53. In contrast, TGF-β acts as a tumor promoter in SBOT cells, which harbor high levels of wild-type p53. These results conflict with previous reports demonstrating that wild-type p53 is required for TGF-β-mediated growth arrest in normal mouse embryonic fibroblasts and hematopoietic progenitor cells (295). In contrast, mutant-p53, but not wild-type p53, is required and can enhance TGF-β-induced breast cancer invasion and metastasis (295, 296). In the SBOT/LGSC system, the TGF-β switch from a tumor promoter to a tumor suppressor may be cell-type specific, or it may be affected by the endogenous level of wild-type p53. Further investigation will be needed to address this question.

A recent study demonstrated that Smad4 loss in colon cancer cells switches TGF-β from a tumor suppressor to a tumor promoter (297). TGF-β induced proliferation, migration, invasion, tumorigenicity and metastasis in Smad4-null colon cancer cells, and these TGF-β-induced oncogenic effects were reversed when Smad4 expression was restored (297). However, it is unknown whether Smad-dependent pathways are required for the observed oncogenic TGF-β effects in Smad4-null cells. Here we demonstrate that the endogenous Smad4 levels were lower in SBOT3.1 compared with MPSC1 cells. Whether the difference in Smad4 expression level affects the TGF-β functions in SBOT3.1 and MPSC1 cells will be an interesting topic for further investigation. Smad4 is essential for many but not all TGF-β-regulated transcriptional responses. In non-canonical signaling pathways, TGF-β activates MAPK, PI3K/Akt and small GTPases that are Smad-independent (183). Our recent study demonstrated that the ERK1/2 and PI3K/Akt pathways are involved in EGF-induced EMT in SBOT cells (291). Here we demonstrated that TGF-β treatment induced ERK1/2 and Akt phosphorylation in SBOT3.1 and MPSC1 cells (Figure 6.8). Because SB431542 is a TβRI inhibitor, it is not surprising that this compound completely blocked the effects of

TGF-β on EMT and invasion in SBOT3.1 cells and apoptosis in MPSC1 cells. However, our results do not exclude the involvement of the non-canonical signaling pathways in TGF-β-induced EMT in SBOT3.1. Thus, further investigation will be needed to address this issue.

We now demonstrate that TGF-β treatment activates Smad3 but not Smad2 in SBOT3.1 and MPSC1 cells. Keratinocytes isolated from Smad2 knockout mice exhibit pathological alterations associated with EMT (298). Furthermore, the absence of Smad2 in these cells leads to greater effects of TGF-β on Snail expression because of the increased availability of the Smad3/Smad4 complexes bound to the Snail promoter (298). Smad2-deficient mouse hepatocytes acquire mesenchymal and promigratory features. In contrast, Smad3-deficient hepatocytes maintain their epithelial characteristics and do not exhibit TGF-β-induced apoptosis. These results suggest that Smad2 may have an antagonistic role in the induction of EMT, whereas Smad3 is required for TGF-β-induced EMT and apoptosis (299).

The transcription factors Snail, Slug, Twist and ZEB1 have been well characterized for their important roles in the regulation of the EMT through decreased E-cadherin expression (122). In addition to their function in down-regulating E-cadherin, Twist and ZEB1 have been shown to up-regulate N-cadherin (134, 141). Snail, Slug and Twist are involved in the TGF-β-induced EMT, which is mainly mediated by Smad3 (298, 300, 301). TGF-β-induced Smad3 binds to the Snail and Slug promoters and activates their transcription (298, 301). TGF-β can not induce Snail expression in renal tubular epithelial cells that are Smad3 deficient (302). TGF-β also induces ZEB1 expression, although Smad3 involvement has not been reported (303). MAPK signaling mediates ZEB1 expression, suggesting that a non-canonical signaling pathway may be involved in TGF-β-induced ZEB1 expression. In this study, TGF-β alone activated Smad3 and induced Snail, Slug, Twist and ZEB1

expression in SBOT3.1 cells, and these effects were abolished by the addition of the TβRI inhibitor SB431542. We have no direct evidence demonstrating that Smad3 is required for the TGF-β-induced expression of these transcription factors. Thus, additional studies will be required to examine the involvement of non-canonical signaling pathways in TGF-β-induced Snail, Slug, Twist and ZEB1 expression in SBOT cells.

In summary, we report for the first time the effects of TGF-β receptor expression in cultured SBOT cells and the LGSC-derived cell line MPSC1. TGF-β treatment induces SBOT3.1 cell invasion by activating EMT. In contrast, TGF-β induces apoptosis in MPSC1 cells. Elucidating the functions of TGF-β in SBOT and LGSC cells will increase our understanding of these particular types of human ovarian cancer.
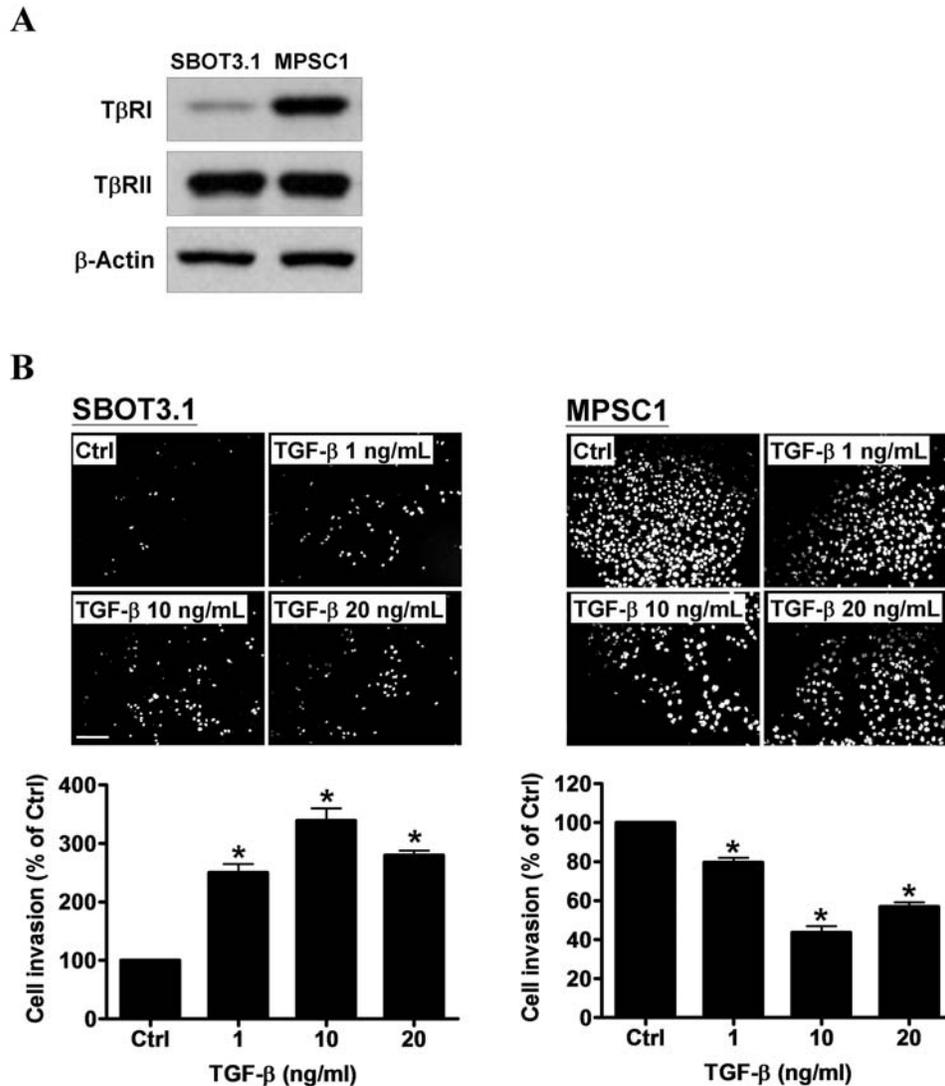
**Figure 6. 1 TGF-β induces SBOT3.1 cell invasion but reduces the invasiveness of MPSC1 cells**

**(A)** The endogenous protein levels of TGF-β type I receptor (TβRI) and type II receptor (TβRII) in SBOT3.1 and MPSC1 cells were analyzed by western blot. **(B)** SBOT3.1 and MPSC1 cells were treated with increasing TGF-β doses (1, 10 and 20 ng/ml) and seeded into Matrigel-coated transwell inserts. After 48 hr incubation, non-invading cells were wiped from the upper side of the filter, and the nuclei of the invading cells were stained with Hoechst 33258. The top panels show representative images of the invasion assays. The scale bar represents 200 μm. The bottom panels show summarized quantitative results, which are expressed as the mean ± SEM of at least three independent experiments. *p<0.05 compared with the control samples (Ctrl).
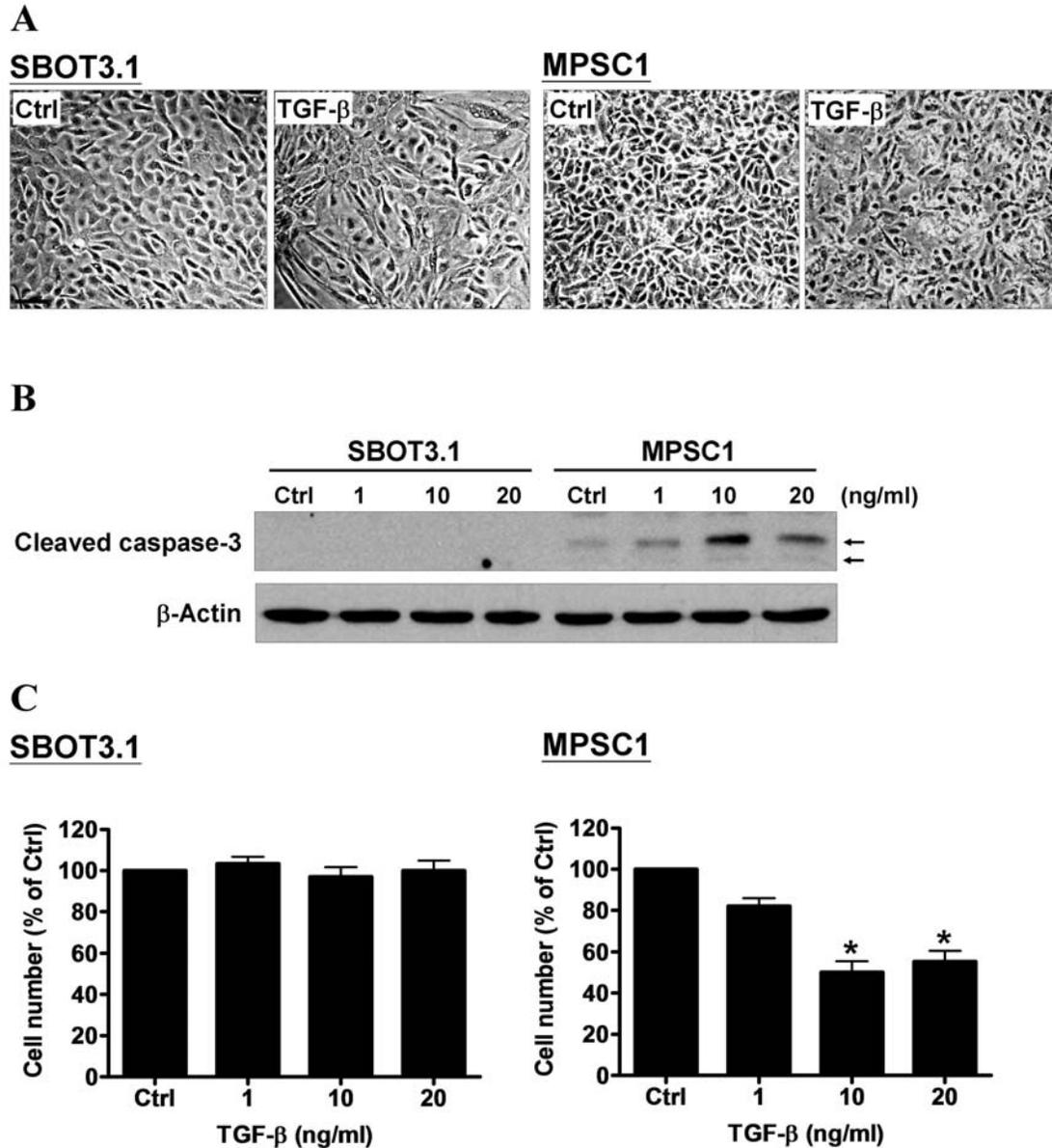
**Figure 6. 2 TGF-β induces apoptosis in MPSC1 but not SBOT3.1 cells**

**(A)** SBOT3.1 and MPSC1 cells were treated without (control; Ctrl) or with 10 ng/ml TGF-β for 48 hr, and the resultant morphology was microscopically examined. The scale bar represents 200 μm. **(B)** SBOT3.1 and MPSC1 cells were treated with increasing TGF-β doses (1, 10 and 20 ng/ml) for 24 hr, and the levels of cleaved caspase-3 were examined using western blot analysis. Arrows indicate cleaved caspase-3. **(C)** SBOT3.1 and MPSC1 cells were treated with increasing TGF-β doses (1, 10 and 20 ng/ml) for 48 hr, and the cell number changes were examined using a trypan blue exclusion assay. *$p<0.05$ compared with the control samples (Ctrl).

133
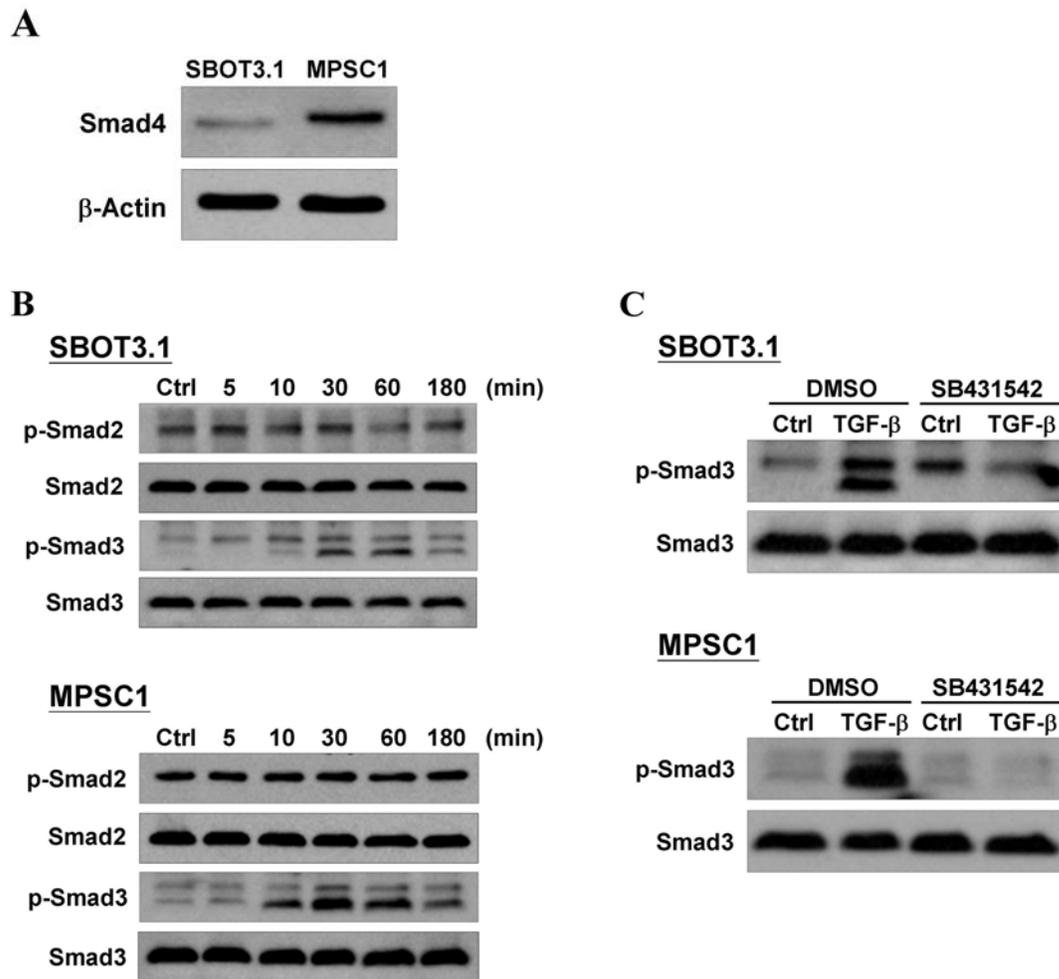
**Figure 6. 3 TGF-β induces the phosphorylation of Smad3 but not Smad2**

**(A)** The endogenous Smad4 protein levels were analyzed in SBOT3.1 and MPSC1 cells using a western blot. **(B)** SBOT3.1 and MPSC1 cells were treated with 10 ng/ml TGF-β for the indicated durations. The Smad2 and Smad3 phosphorylation levels were determined using western blot analysis with antibodies specific for the phosphorylated, activated forms of Smad2 (p-Smad2) and Smad3 (p-Smad3). The membranes were stripped and reprobed with antibodies directed against Smad2 and Smad3. **(C)** SBOT3.1 and MPSC1 cells were treated with SB431542 (10 μM) in the presence or absence of 10 ng/ml TGF-β for 30 min. The Smad2 and Smad3 phosphorylation levels were analyzed by western blot.

# A

## SBOT3.1



## MPSC1



# B

## SBOT3.1



## MPSC1

**C**

**MPSC1**



**D**

**ILGC**

E
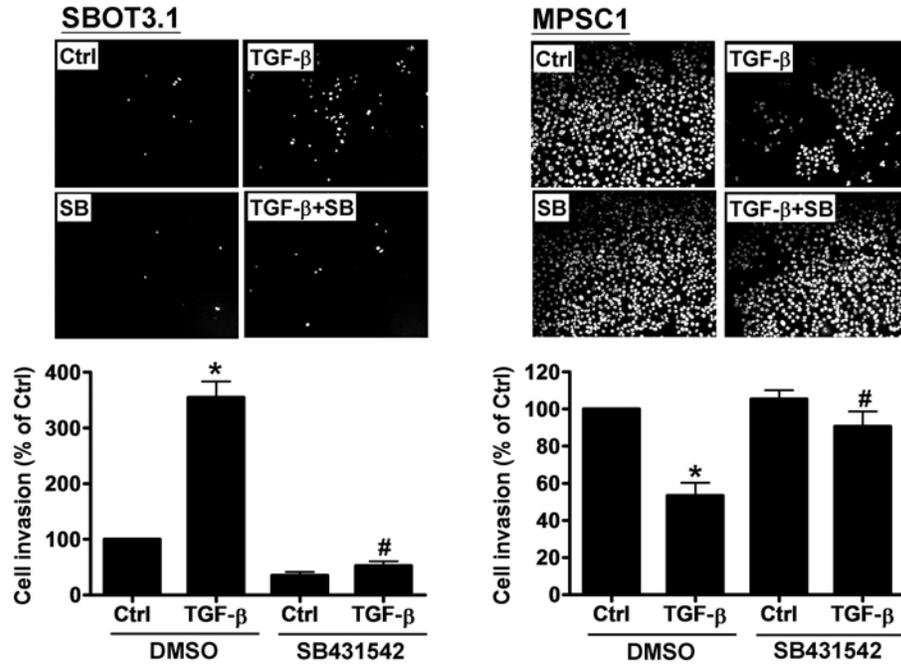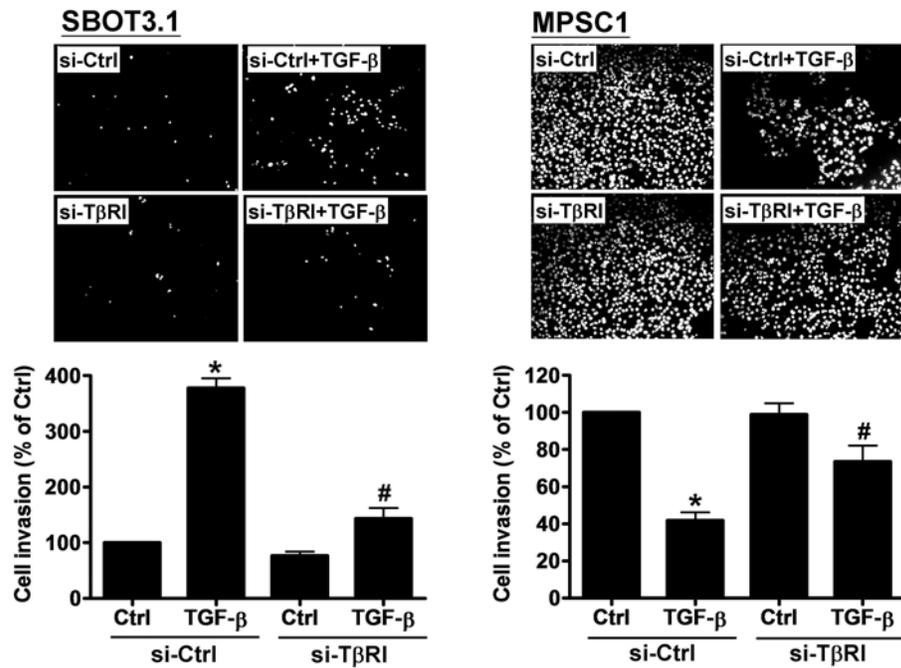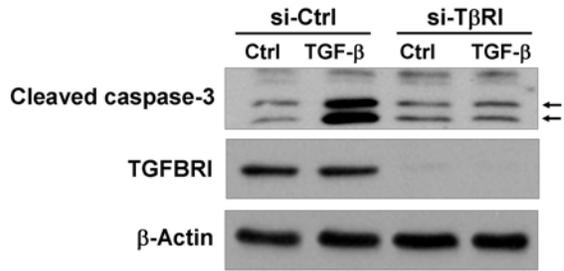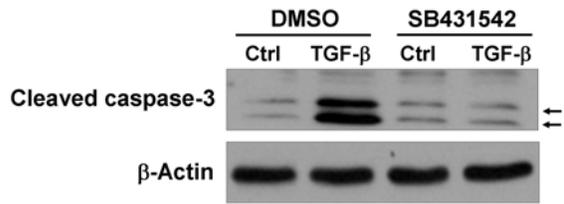


MPSC1

ILGC
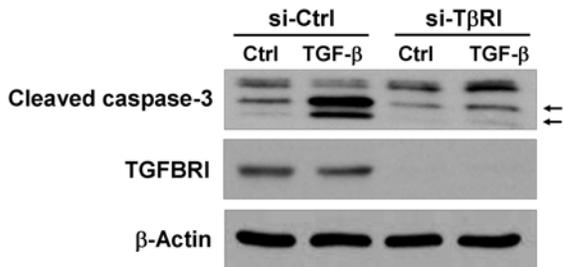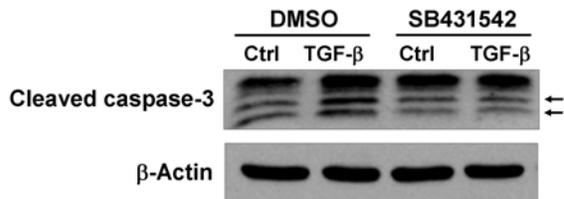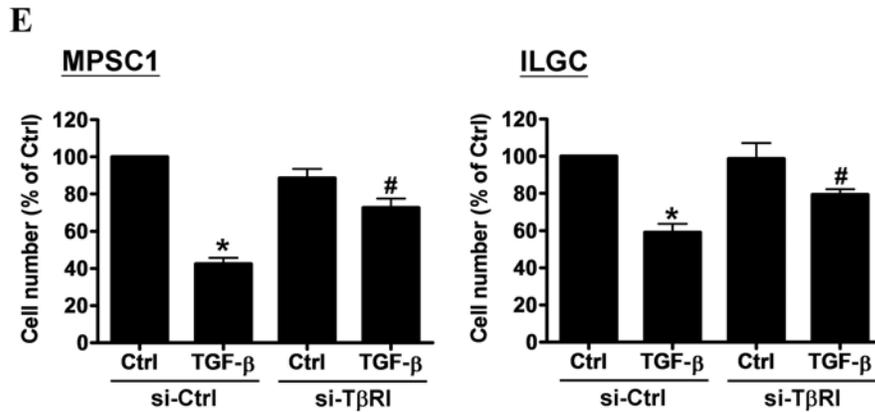
**Figure 6. 4 The specific TβRI inhibitor, SB431542, and TβRI siRNA abolished TGF-β-induced cell invasion and apoptosis**

**(A)** SBOT3.1 and MPSC1 cells were treated with 10 ng/ml TGF-β in combination with SB431542 (10 μM). **(B)** SBOT3.1 and MPSC1 cells were transfected with 50 nM control siRNA (si-Ctrl) and TβRI siRNA (si-TβRI), and after 48 hr, the cells were treated with 10 ng/ml TGF-β. The cells were seeded onto Matrigel-coated transwell inserts. After 48 hr incubation, the non-invading cells were wiped from the upper side of the filter, and the nuclei of the invading cells were stained with Hoechst 33258. The results are expressed as the mean ± SEM of at least three independent experiments. **(C)** MPSC1 cells were treated with 10 ng/ml TGF-β in combination with SB431542 (10 μM) for 24 hr or transfected with 50 nM control siRNA (si-Ctrl) or TβRI siRNA (si-TβRI) for 48 hr and then treated with 10 ng/ml TGF-β for 24 hr, and the levels of cleaved caspase-3 were examined western blot. Arrows indicate cleaved caspase-3. **(D)** ILGC cells were treated with 10 ng/ml TGF-β in combination with SB431542 (10 μM) for 24 hr or transfected with 50 nM control siRNA (si-Ctrl) or TβRI siRNA (si-TβRI) for 48 hr and then treated with 10 ng/ml TGF-β for 24 hr, and the levels of cleaved caspase-3 were examined western blot. Arrows indicate cleaved caspase-3. **(E)** MPSC1 and ILGC cells were transfected with 50 nM control siRNA (si-Ctrl) or TβRI siRNA (si-TβRI) for 48 hr and then treated with 10 ng/ml TGF-β for 48 hr, and the changes of cell number were examined using trypan blue exclusion assay. *$p<0.05$ compared with Ctrl in the DMSO group or Ctrl in the si-Ctrl group. #$p<0.05$ compared with TGF-β in the DMSO group or TGF-β in the si-Ctrl group.
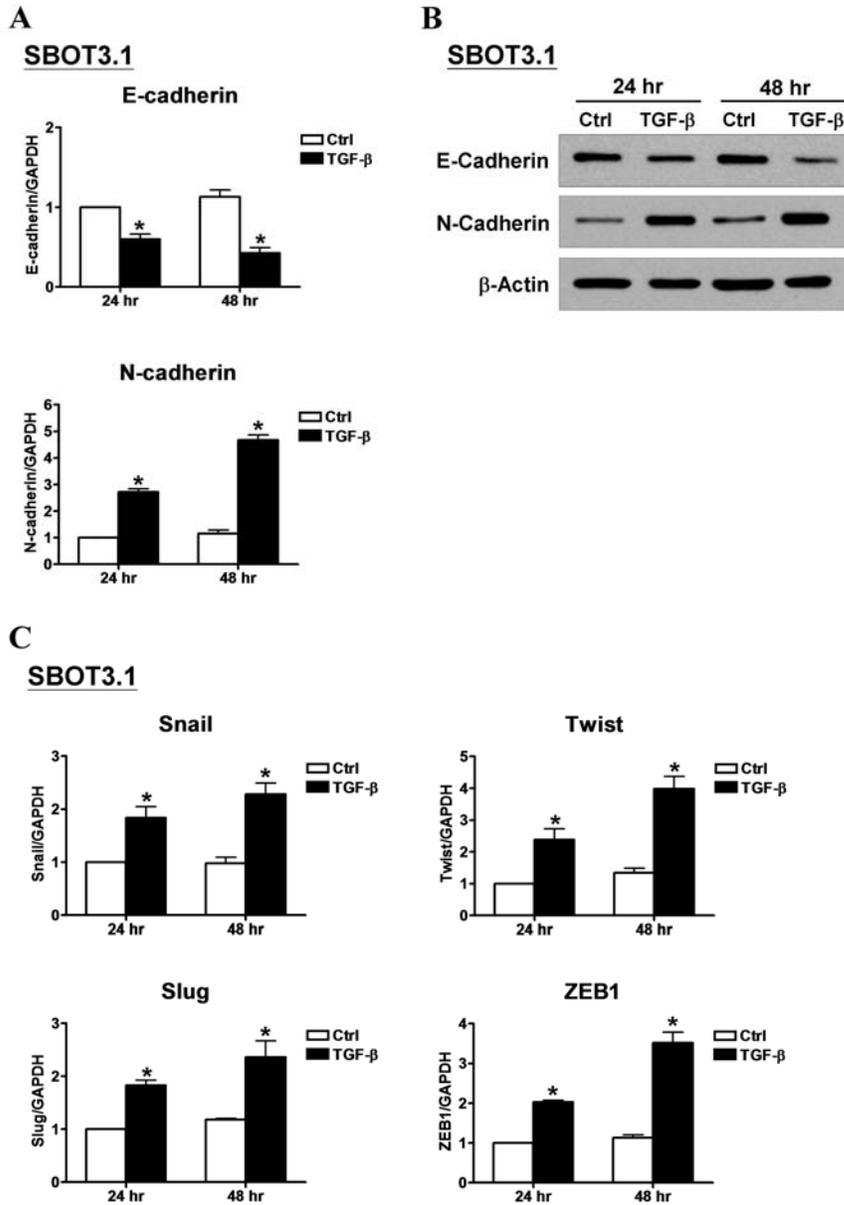
**Figure 6. 5 TGF-β induces a switch from E- to N-cadherin in SBOT3.1 cells**
**(A)** SBOT3.1 cells were treated with 10 ng/ml TGF-β for 24 and 48 hr. The E-cadherin and N-cadherin mRNA levels were analyzed by RT-qPCR. **(B)** SBOT3.1 cells were treated with 10 ng/ml TGF-β for 24 and 48 hr, and the E-cadherin and N-cadherin protein levels were analyzed by western blot. **(C)** SBOT3.1 cells were treated with 10 ng/ml TGF-β for 24 and 48 hr, and the Snail, Slug, Twist and ZEB1 mRNA levels were analyzed by RT-qPCR. The RT-qPCR results are expressed as the mean ± SEM of at least three independent experiments. *p<0.05 compared with time-matched control samples (Ctrl).

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**A**

SBOT3.1

**E-cadherin**

SBOT3.1

**N-cadherin**

**B**

SBOT3.1

TβRI

E-cadherin

N-cadherin

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**C**
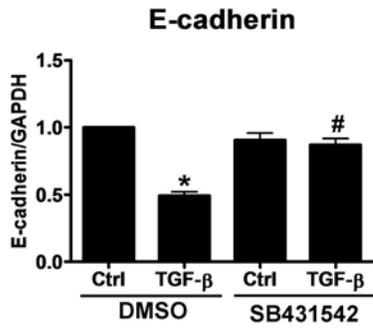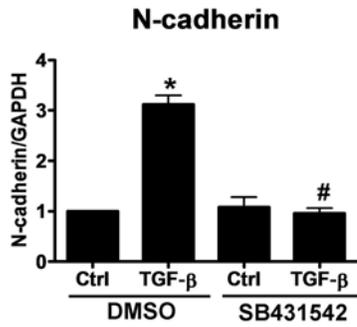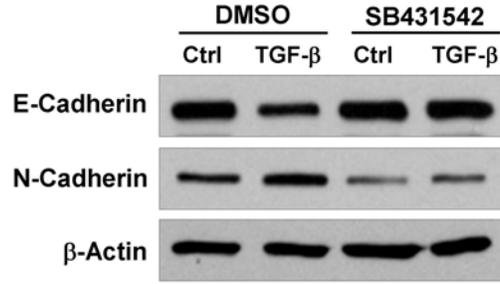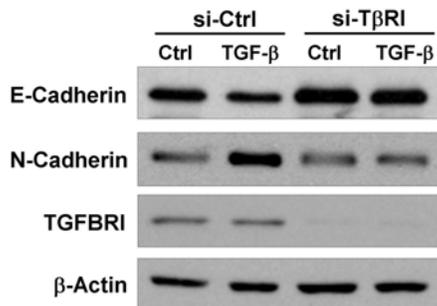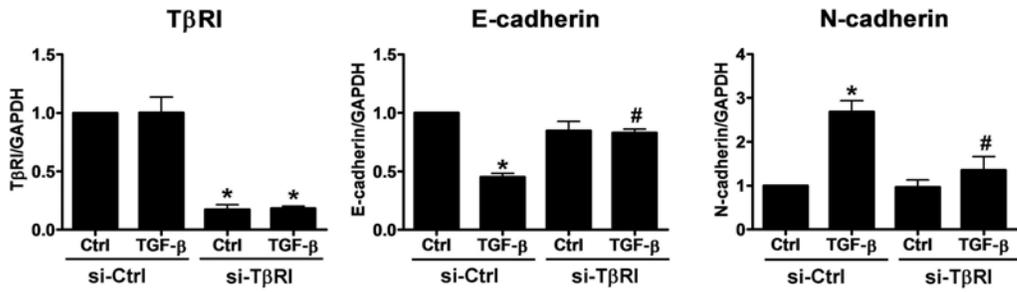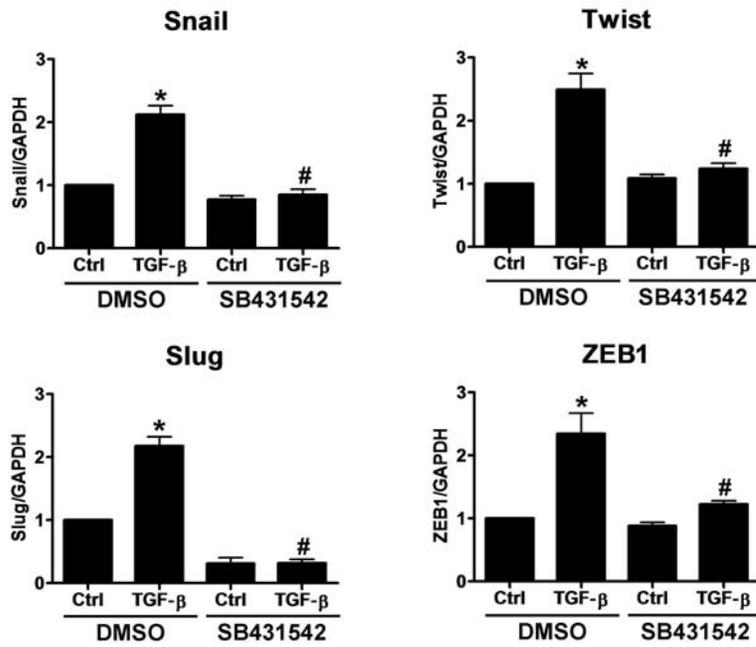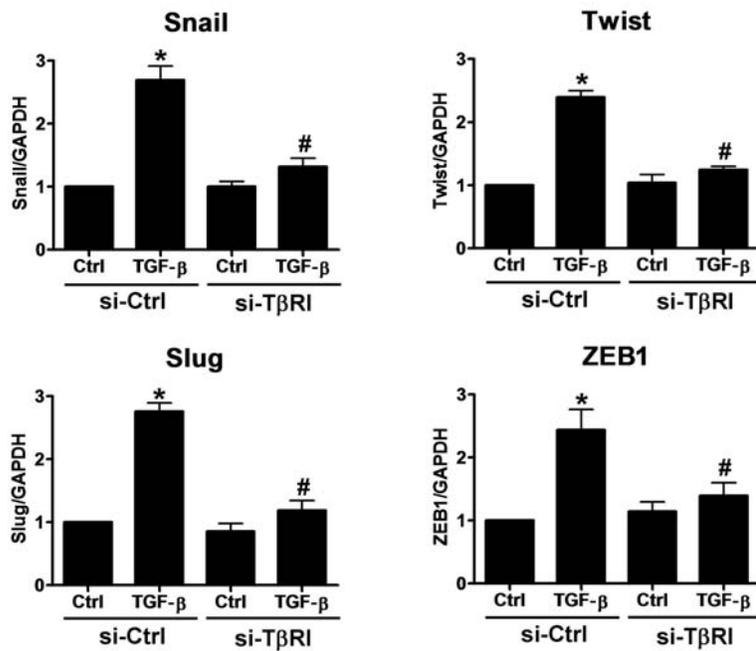
<u>SBOT3.1</u>

**Figure 6. 6 The specific TβRI inhibitor, SB431542, and TβRI siRNA abolished the TGF-β-induced differential change in E- and N-cadherin expression and the TGF-β-induced effects on Snail, Slug, Twist and ZEB1 expression**

**(A)** SBOT3.1 cells were treated with SB431542 (10 μM) in the presence or absence of 10 ng/ml TGF-β. E-cadherin and N-cadherin mRNA and protein levels were analyzed by RT-qPCR and western blot, respectively. **(B)** SBOT3.1 cells were transfected with 50 nM control siRNA (si-Ctrl) or TβRI siRNA (si-TβRI), and after 48 hr, the cells were treated with 10 ng/ml TGF-β. The TβRI, E-cadherin and N-cadherin mRNA and protein levels were analyzed by RT-qPCR and western blot, respectively. **(C)** SBOT3.1 cells were treated with SB431542 (10 μM) in the presence or absence of 10 ng/ml TGF-β. The Snail, Slug, Twist and ZEB1 mRNA levels were analyzed by RT-qPCR. **(D)** SBOT3.1 cells were transfected with 50 nM control siRNA (si-Ctrl) or TβRI siRNA (si-TβRI), and after 48 hr, the cells were treated with 10 ng/ml TGF-β. The Snail, Slug, Twist and ZEB1 mRNA levels were analyzed by RT-qPCR. The RT-qPCR results are expressed as the mean ± SEM of at least three independent experiments. *$p<0.05$ compared with the Ctrl in the DMSO group or the Ctrl in the si-Ctrl group. [#]$p<0.05$ compared with TGF-β in the DMSO group or TGF-β in the si-Ctrl group.
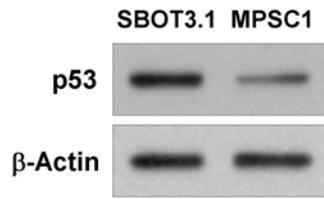
**Figure 6. 7 The endogenous p53 protein levels were analyzed in SBOT3.1 and MPSC1 cells using a western blot**
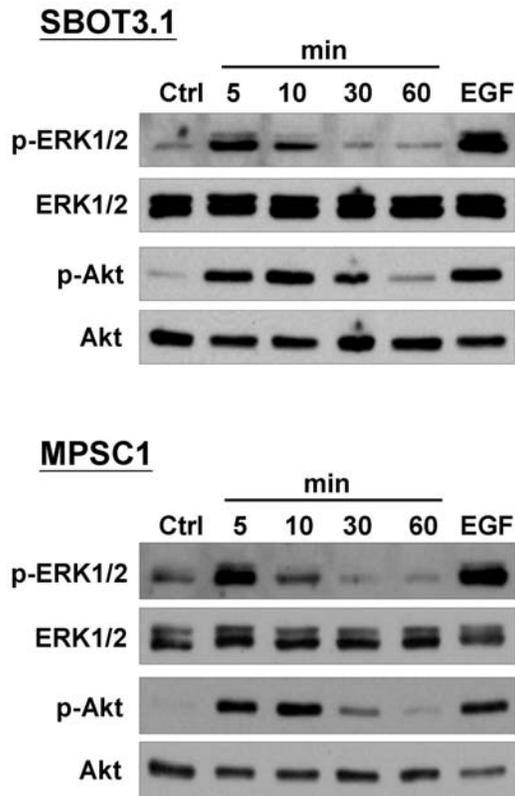
**Figure 6. 8 TGF-β induces the phosphorylation of ERK1/2 and Akt in SBOT3.1 and MPSC1**

Cells were treated with 10 ng/ml TGF-β for the indicated durations. The ERK1/2 and Akt phosphorylation levels were determined using western blot analysis with antibodies specific for the phosphorylated, activated forms of ERK1/2 (p-ERK1/2) and Akt (p-Akt). The membranes were stripped and reprobed with antibodies directed against ERK1/2 and Akt. Cells treated with EGF for 30 min were used as positive control.

# Chapter 7 Conclusion

## 7.1 Summary

Low-grade serous ovarian carcinomas (LGSC) and its precursor serous borderline ovarian tumors (SBOT) are rare diseases. At a clinical, cellular and molecular level, SBOT and LGSC have been considered distinct entities that are unrelated to high-grade serous ovarian carcinomas (HGSC). To date, the vast majority of research has been focused on HGSC, which is the most common ovarian malignancy. In contrast, because very few experimental systems have been available, little is known about the nature of SBOT, which are non-invasive and have a better prognosis than the invasive serous ovarian tumors (200). However, a large proportion of SBOT recur as invasive LGSC, which is associated with a significantly worse prognosis (200). Therefore, studying the mechanisms underlying the progression from SBOT to invasive carcinomas may help us to identify and predict those tumors that will progress to a more deadly form of the disease.

Our laboratory has established a novel cell culture model for human SBOT in which many characteristics of SBOT that are observed clinically are retained in culture (48). Among these established SBOT cell lines, the SBOT3.1 cells represent a spontaneously permanent cell line, whereas SBOT4 cells have a limited life span but can be propagated for approximately 10-12 population doublings. These SBOT cells do not exhibit characteristics normally associated with neoplastic transformation, including invasiveness, anchorage-independent growth and tumorigenicity (48). In addition, we have obtained the LGSC-derived cell line, MPSC1, from Dr. Ie-Ming Shih at Johns Hopkins University. MPSC1 cells exhibit the *BRAF* mutation, high motility and invasiveness characteristic of invasive LGSC (208, 291). These cell lines therefore provide a novel experimental model to study the effects of genetic alterations and the influence of growth factors on the progression

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of non-invasive SBOT to invasive LGSC.

The studies from our laboratory and from other groups have suggested that the down-regulation of p53 expression may be involved in the progression from SBOT to LGSC (54, 198). As shown in the results from Chapter 3, by using loss- and gain-of-function approaches, I first demonstrated that either the inhibition of p53 functions by SV40 LT or the siRNA-mediated knockdown of p53-induced SBOT cell invasion. In contrast, the overexpression of p53 attenuated the invasiveness of MPSC1 cells. These results confirmed the hypothesis of the previous study that suggested the progression from non-invasive SBOT to invasive LGSC may involve the attenuation of p53 signaling (54). Given the known negative regulatory effect of p53 on the expression of the PI3K catalytic subunit p110α (encoded by *PIK3CA*) (206) in HGSC, I also examined the possible involvement of PI3K/Akt signaling in p53-regulated SBOT and MPSC1 cell invasion. Similar to the results obtained from HGSC, the inhibition of p53 up-regulated p110α expression and subsequently enhanced the PI3K/Akt signaling. In contrast, the overexpression of p53 in MPSC1 cells yielded opposite results. These results are part of the increasing evidence indicating that the activation of PI3K/Akt signaling is a central feature of EMT (185). In addition, our previous study suggested that SV40 LT/ST-induced EMT may contribute to the increase of SBOT cell invasion (198). Therefore, I thought to connect p53-regulated PI3K/Akt singling to EMT. I found that the inhibition of p53 enhanced PI3K/Akt signaling, which up-regulated Slug and Twist and led to the down-regulation of E-cadherin. Finally, this down-regulation of E-cadherin contributed to SBOT cell invasion. Conversely, in invasive MPSC1 cells, enhancing the levels of p53 decreased cell invasion and diminished the PI3K/Akt-mediated down-regulation of E-cadherin. Interestingly, the inhibition of Rb also enhanced invasiveness but did not affect the levels of *PIK3CA* or E-cadherin in SBOT cells, suggesting that it acts

through a different pathway. To our knowledge, these results are the first to show that p53 has an important role in the progression from SBOT to invasive LGSC. In addition, our findings suggest that the down-regulation of E-cadherin by the PI3K/Akt pathway contributes to this progression.

Aberrant DNA methylation has been detected in many types of human cancers and has been reported to be involved in the progression from non-invasive to invasive ovarian carcinomas (237-239). In Chapter 4, I tested the hypothesis that the inhibition of p53 down-regulates E-cadherin by regulating the methylation of its promoter in SBOT cells. I showed that DNA methyltransferase 1 (DNMT1) was increased in SV40 LT-infected SBOT4, SBOT4-LT, and MPSC1 cells. Treatment with 5-Aza-dC, a DNMT1 inhibitor, restored E-cadherin promoter methylation and expression and inhibited cell invasion in the invasive SBOT4-LT and MPSC1 cells. Moreover, the knockdown of p53 in SBOT cells induced DNMT1 expression and led to an increase in E-cadherin promoter methylation. Additionally, the activation of PI3K/Akt signaling was required for p53 inhibition-induced DNMT1 expression. The increase in DNMT1 was associated with the inhibition of the p53-induced down-regulation of E-cadherin and cell invasion. These findings reveal an important role for p53 in the progression of SBOT to an invasive LGSC and suggest that the down-regulation of E-cadherin by DNMT1-mediated promoter methylation contributes to this process.

Taken together, the results from Chapters 3 and 4 demonstrate that p53 acts as a tumor suppressor in the regulation of SBOT and LGSC cell invasion by regulating E-cadherin expression through PI3K/Akt-mediated transcriptional and epigenetic machineries.

As mentioned previously, epidermal growth factor (EGF) and transforming growth factor-beta (TGF-β) play important roles in the regulation of EMT and cell invasion in HGSC (276). However, their function in the regulation of EMT and cell invasion in SBOT and

LGSC cells remain unknown. Therefore, in Chapter 5, I investigated the effect of EGF on SBOT and LGSC cell invasion. I showed that the EGF receptor was expressed and that EGF treatment increased cell migration and invasion in SBOT and LGSC cells. However, EGF induced the down-regulation of E-cadherin and concurrent up-regulation of N-cadherin in SBOT cells, but not in LGSC cells. In SBOT cells, the expression of the transcriptional repressors of E-cadherin, Snail, Slug and ZEB1, was increased by EGF treatment. Treatment with EGF led to the activation of downstream ERK1/2 and PI3K/Akt signaling. The inhibition of ERK1/2 diminished the EGF-induced cadherin switch and the up-regulation of Snail, Slug and ZEB1 and an EGF-mediated increase in SBOT cell migration and invasion. The inhibition of PI3K/Akt signaling had similar effects but could not block the EGF-induced up-regulation of N-cadherin and ZEB1.

In Chapter 6, I demonstrated the effects of TGF-β on SBOT and MPSC1 cells, which express TGF-β type I and type II receptors. TGF-β treatment induced the invasiveness of SBOT cells but reduced the invasiveness of MPSC1 cells. Apoptosis assays revealed that TGF-β induced apoptosis in MPSC1 cells but not in SBOT cells. TGF-β treatment led to the activation of Smad3 but not Smad2. The inhibition of TβRI abolished the SBOT invasion induced by TGF-β, and it prevented TGF-β-induced apoptosis in MPSC1 cells. In SBOT cells, we showed that TGF-β treatment down-regulated E-cadherin and concurrently up-regulated N-cadherin. In addition, TGF-β treatment increased the expression of Snail, Slug, Twist and ZEB1. In contrast, the inhibition of TβRI abolished the effects of TGF-β on the relative cadherin expression levels and those of Snail, Slug, Twist and ZEB1.

Taken together, the results from Chapters 5 and 6 demonstrated that EGF and TGF-β induce SBOT cell invasion by activating EMT. Interestingly, these results suggest that there are EMT-independent mechanisms that mediate EGF-induced LGSC cell invasion. Moreover,

we also demonstrated dual TGF-β functions: the induction of SBOT cell invasion by EMT activation and the promotion of apoptosis in LGSC cells (Figure 7.1).

## 7.2 Discussion

### 7.2.1 Does invasive SBOT represent invasive LGSC?

In this study, I demonstrated that the direct knockdown of E-cadherin induced SBOT cell invasion. In contrast, the overexpression of E-cadherin attenuated cell invasion in MPSC1 cells. These results indicate that E-cadherin levels are an important factor that regulates SBOT and LGSC cell invasion. Although I have performed experiments by using loss- and gain-of-function approaches for E-cadherin in both SBOT and the LGSC-derived cell line, MPSC1, I am aware that the change from non-invasive to the invasive phenotype in SBOT cells or *vice versa* in MPSC1 cells may not fully represent the progression from non-invasive SBOT to invasive LGSC.

In addition to different levels of p53 and p53-related genes, microarray results from a previous study also showed that many other genes displayed significant differences between SBOT and LGSC (54). For example, the expression of Moesin (encoded by the *MSN* gene) were significantly higher in SBOT than in LGSC (54). Ezrin, Radixin and Moesin belong to the ERM family, which were originally characterized as actin-binding proteins that function as linkers between the actin cytoskeleton and plasma membrane proteins. ERM proteins have been shown to regulate epithelial cell morphogenesis, adherens junctions, cell migration, invasion and metastasis (304). In addition, Moesin could maintain epithelial integrity by antagonizing the Rho pathway, and the down-regulation of Moesin was associated with the loss of epithelial characteristics and high invasiveness (305). Therefore, these findings would support our hypothesis that progression from non-invasive SBOT to invasive LGSC is

regulated by multiple factors and that E-cadherin may only partially mediate this progression.

**7.2.2 What is the major role of p53 in the regulation of SBOT cell invasion?**

In the process of metastasis, tumor cells must first dissociate from the primary tumor and acquire motility and proteolytic abilities before they can invade the stroma to reach the blood or lymph vessels and finally metastasize to other tissues. At present, the function of p53 protein has been extensively studied, making p53 perhaps the best known of all tumor suppressors. In this study, I demonstrated that the inhibition of p53 induced SBOT cell invasion by down-regulating E-cadherin expression. The major function of E-cadherin is to maintain cell-cell adhesion. Similar to other cancer cells, the loss of cell-cell adhesion in SBOT cells may directly contribute to cell invasion. However, it is not surprising that the inhibition of p53 can induce SBOT cell invasion by affecting protease activity and cell motility.

A recent study in human breast and prostate cancer cells demonstrated that Twist directly interacted with the DNA-binding domain of p53 and suppressed the DNA-binding activity of p53 (306). This study indicated that p53 acts as an EMT repressor and that when Twist is up-regulated through stimulation by growth factors, the loss of EMT suppression by p53 may contribute to the induction of EMT. In addition to Twist, a recent study in human lung cancer cells showed that the expression of wild-type p53 could enhance MDM2-mediated protein degradation of Slug, which led to the up-regulation of E-cadherin and a decrease in cell invasion (215). In this study, I showed that the inhibition of p53 increased Slug and Twist expression by enhancing PI3K/Akt signaling. It will be interesting to further examine whether p53 can directly regulate Slug and Twist through mechanisms similar to those reported in other cancer cells.

Mutations in p53 are the most common genetic abnormalities in most human cancers and occur predominantly through missense mutations that result in the accumulation of mutant p53 protein. These p53 mutations have been shown to cause gain-of-function activities (307). In addition to its well-known functions in the regulation of cell proliferation and apoptosis, there is increasing evidence that p53 can regulate cancer metastasis by affecting cell migration and invasion (94). In the past few years, many studies have shown that gain-of-function mutant p53 proteins induced an aggressive cancer phenotype. Moreover, at the molecular level, mutant p53 increased tumor cell proliferation, migration and invasion by regulating Rho GTPases and induced EMT by up-regulating Twist expression (296, 308-312). Mutations in p53 are very rare in SBOT and LGSC (10). We have confirmed that SBOT3.1 cells harbor wild-type p53. These results indicate that the effect of p53 on SBOT invasion was not due to a mutation-mediated gain-of-function.

We previously showed that SBOT cells expressed MMP2, MMP9 and variable levels of active uPA and that the induction of invasion in SV40 LT/ST-transfected SBOT cells was not associated with changes in protease secretion (198). Therefore, the inhibition of p53 appears to increase cell motility but does not modulate protease activity. Indeed, recent studies have reported that the loss of wild-type p53 could enhance cell motility in a manner that can contribute to cancer cell invasion and metastasis. I have examined the expression of polymerized actin, F-actin, by using rhodamine-conjugated phalloidin staining. The preliminary results showed that SBOT4 cells exhibited obvious cortical actin and small amounts of F-actin localized within the cytoplasm. Consistent with the morphological change, F-actin was significantly increased and more dispersed within the cytoplasm in SV40 large T antigen-infected cells, SBOT4-LT (Figure 7.2). These results support the previous findings that the inhibition of p53 or Rb increases cell motility by regulating Rho GTPase-mediated

cytoskeleton organization (98, 266).

**7.2.3 Is the down-regulation of E-cadherin necessary to induce SBOT cell invasion?**

In many types of human epithelial cancers, including ovarian cancer, E-cadherin expression is down-regulated or lost. Numerous *in vitro* studies have supported the role of E-cadherin as an invasion suppressor (119). However, there is emerging evidence that during cancer progression, the expression levels of E-cadherin may be dynamic and highly context-dependent (313). E-cadherin-negative human breast cancer cell lines were not more invasive than E-cadherin-positive cell lines (314). The breast cancer cell line BT-20N expressed high levels of E-cadherin and was highly motile and invasive (118). Moreover, the overexpression of E-cadherin did not alter the morphology or invasiveness of invasive breast cancer cell lines (315). In this study, I showed that the siRNA-mediated knockdown of E-cadherin increased the number of invaded SBOT cells through Matrigel, which directly indicated its role in the regulation of cell invasion. Taken together, these findings suggest that E-cadherin may play a role in the suppression of SBOT cell invasion. In this context, the regulation of invasiveness in other cancer cells appears to depend on other factors, in addition to E-cadherin.

The loss of E-cadherin is likely to affect tumorigenesis in many ways, which increases the complexity of the process through which the loss of E-cadherin promotes tumor progression. Many potential mechanisms have been reported to be involved in the loss of E-cadherin-induced tumorigenesis (316). Among these mechanisms, the induction of β-catenin signaling is known to contribute to the loss of E-cadherin-induced tumorigenesis, including cell proliferation and invasion (317). Our preliminary results showed that in SBOT4 cells, β-catenin was localized in the cell-cell junctions, whereas in SV40 large T

antigen-infected cells, SBOT4-LT, the majority of β-catenin was translocated into the nucleus (Figure 7.3). In addition, our recent study indicated that E-cadherin inhibits high-grade ovarian cancer cell growth by suppressing PI3K/Akt signaling via β-catenin-Egr1-mediated PTEN expression (318). Therefore, future study will be needed to address the role of β-catenin in the regulation of SBOT cell proliferation and invasion.

**7.2.4 What is the possible mechanism that mediates EMT-independent cell migration and invasion in LGSC?**

To our surprise, unlike what has been previously observed for SBOT cells, treatment with EGF did not affect the levels of E-cadherin and N-cadherin but increased cell migration and invasion in LGSC-derived MPSC1 cells, which indicates the involvement of an EMT-independent pathway. These findings corroborate a previous study showing a significant difference in gene expression between SBOT and LGSC, which indicates that these two types of tumors may exhibit different biological behaviors (54).

Rather than migrating or invading individually, cancer cells can migrate as a cohesive group (319). This type of cell migration is called "collective migration" and is an important mechanism that is involved in the regulation of morphogenesis and wound repair (319). In addition to its roles in the regulation of normal physiological functions, collective migration contributes to cell invasion in many types of cancers, although the molecular mechanisms of collective migration/invasion of cancer cells are not well characterized (320). In collective migration, cadherin proteins can be rapidly relocalized, which allows changes of cell position in the cohesive group (320). Moreover, cadherin-mediated cell-cell adhesion is important for the maintenance of collective polarity and guiding the direction of movement (320). Therefore, EMT-independent cancer migration and invasion can occur in the LGSC cells.

Indeed, many studies have shown that EMT or down-regulation of E-cadherin is not necessary for the cell migration and invasion of certain types of human cancers such as prostate and breast cancers (118, 313, 321). The results from those studies demonstrated that E-cadherin was still expressed in the invasive and malignant tumor cells and that full EMT is rarely detected in tumor biopsies (118, 313, 321). In human breast cancer and mouse pancreatic tumor cells, overexpression of podoplanin, a mucin-type transmembrane glycoprotein, induced cell migration and invasion by relocalizing ERM proteins and rearranging the actin cytoskeleton without altering EMT markers (322). In other types of cancer cells, including high-grade serous ovarian cancer, EGF increased cell motility by regulating actin cytoskeleton remodeling, and MMP activity is required for EGF-induced cell invasion (261, 262, 323, 324). Taken together, these studies indicate that the regulation of the actin cytoskeleton and ECM remodeling may contribute to the EMT-independent cell migration and invasion. However, further investigation will be needed to define the EMT-independent mechanism that mediates EGF-induced LGSC cell migration and invasion.

**7.2.5 What is the biological function of N-cadherin in SBOT cells?**

In other epithelial cancer cells, N-cadherin can promote cell motility and invasion (117, 325). In breast cancer cells, overexpression of N-cadherin promoted cell motility and invasion regardless of the level of E-cadherin expression (118). In this study, I showed that the inhibition of p53 and treatment with EGF or TGF-β down-regulated E-cadherin and concurrently up-regulated N-cadherin in SBOT cells. However, we did not directly investigate the role of N-cadherin in the regulation of SBOT cell invasion. Our previous study has shown that overexpression of N-cadherin did not alter E-cadherin levels and was not sufficient to generate an invasive phenotype in SBOT cells (198). These findings do not

rule out the involvement of N-cadherin in the regulation of SBOT cell invasion because we did not directly apply N-cadherin siRNA to examine the requirement for N-cadherin in the inhibition of p53 or EGF/TGF-β-induced cell invasion. It is possible that the exogenous expression of N-cadherin alone is insufficient to promote SBOT cell invasion, although N-cadherin may potentiate cell invasion induced by other co-expressed factors.

N-cadherin has been shown to play an important role in the regulation of cell proliferation, apoptosis, migration and differentiation (326). N-cadherin also can connect to the actin cytoskeleton via α-catenin and β-catenin. Engagement of N-cadherin regulates cell-cell adhesion by activating the RhoGTPase Rac1 and the non-receptor tyrosine kinase Fer (327). Moreover, N-cadherin also interacts with several growth factor receptors, such as PDGFR and FGFR, which sustains the activation of MAPK pathway and contributes to increased cell motility, invasion and MMP secretion (119). Therefore, it is clear that N-cadherin is actively involved in regulating cell motility by modulating actin cytoskeleton remodeling and growth factor signaling. However, these pleiotropic functions of N-cadherin in SBOT cells warrant future experimental investigations.

## 7.2.6 What is the major signaling mechanism that mediates the down-regulation of E-cadherin in SBOT cells?

The MAPK, PI3K/Akt and Smad signaling pathways mediate the induction of EMT through many growth factors (166, 167). In high-grade ovarian cancer cell lines, I found that EGF treatment resulted in the activation of EGFR and induced $H_2O_2$ production and p38 MAPK activation to increase the expression of Snail, which subsequently resulted in the down-regulation of E-cadherin. Additionally, EGF engaged the EGFR-activated ERK1/2 and PI3K/Akt signaling pathways, which subsequently induced the expression of Egr-1. Activated

Egr-1 increased the expression of Slug to down-regulate E-cadherin, which contributed to EGF-induced cell invasion (197, 328). Compared with HGSC, EGF activated different signaling pathways in SBOT and LGSC. I showed that, similar to HGSC, EGF activated both ERK1/2 and PI3K/Akt signaling pathways in SBOT and LGSC cells, whereas p38 MAPK was only activated in LGSC cells. Because $H_2O_2$ production mediates the EGF-induced activation of p38 MAPK, these results suggest that the lack of effect of EGF on p38 MAPK activation in SBOT cells likely reflects an uncoupling of EGFR activation from $H_2O_2$ production.

Many studies have revealed the complexity of the signaling pathways that mediate the EMT process (166, 167). After TGF-β treatment, my results corroborated other findings that the Smad-dependent pathway was required for TGF-β-induced EMT (177). Interestingly, my results also demonstrated that TGF-β treatment induced ERK1/2 and Akt activation in SBOT cells, which elucidated the involvement of the MAPK and PI3K/Akt signaling pathways in the TGF-β-induced down-regulation of E-cadherin. Based on results from other laboratories, as well as our own results, I believe that there are different signaling pathways that are involved the down-regulation of E-cadherin in response to growth factors. Among those signaling pathways, MAPK, PI3K/Akt and Smad pathways may partially contribute to the down-regulation of E-cadherin. Further study will be needed to delineate this complex signaling network that is involved in the regulation of EMT.

## 7.3 Limitations of this study and future directions

I am aware that all of the experiments presented in this study were performed in an *in vitro* system. I did not have *in vivo* data to support these findings due to the lack of an animal model and the rarity of clinical samples resulting from the low incidences of SBOT and

LGSC. However, our *in vitro* model allowed me to investigate the detailed molecular mechanisms that mediate SBOT and LGSC cell invasion. For example, I can use pharmacological inhibitors, overexpression or siRNA to examine the role of specific signaling pathways or molecules of interest. These approaches will be difficult or impossible to perform in an *in vivo* model. Therefore, I believe that my study will provide pioneering information for future experimental designs.

In the future, our laboratory will cooperate with Dr. Blake Gilks (Department of Pathology and Laboratory Medicine, University of British Columbia) to collect tumor samples from SBOT and LGSC patients. A tissue microarray will be performed to confirm the molecular differences between SBOT and LGSC that have been reported in this study. In addition, Dr. Yuzhuo Wang's group (Department of Experimental Therapeutics, BC Cancer Agency) has established a mouse model of subrenal capsule xenografts of primary human ovarian tumors (47). Thus far, they have successfully generated one SBOT xenograft (47). Our laboratory will cooperate with them and attempt to generate a xenograft for LGSC. This unique model will provide us with a valuable chance to study the biology of SBOT and LGSC *in vivo* and will help us to further identify new therapeutic targets.

As discussed previously, many questions can be easily addressed by using our established *in vitro* systems. The following experiments are suggestions for future directions:

1. To study the molecular mechanism that mediates p53-regulated cytoskeleton remodeling in SBOT cells.

2. To examine the role of Rb in SBOT cell invasion.

3. To investigate the role of N-cadherin in SBOT cell proliferation and invasion.

4. To further delineate the signaling pathways (*i.e.*, β-catenin, Smad-independent and Rho GTPases pathways) that are involved in the regulation of EMT in SBOT cells.

5. To investigate the EMT-independent mechanism that is involved in EGF-induced LGSC cell migration and invasion.

## 7.4 Conclusion

In conclusion, this study has strongly implicated E-cadherin as a tumor suppressor in serous borderline ovarian tumors that acts as an inhibitor of cell invasion. Down-regulation of E-cadherin in serous borderline ovarian tumors may be a critical step that enhances the transition to invasive low-grade serous carcinoma cells (Figure 7.4). Our discoveries using a novel cell culture model for human SBOT are innovative and not only help us to better understand this rare disease but also break new ground for other investigators to delve into this uncharted area of research.
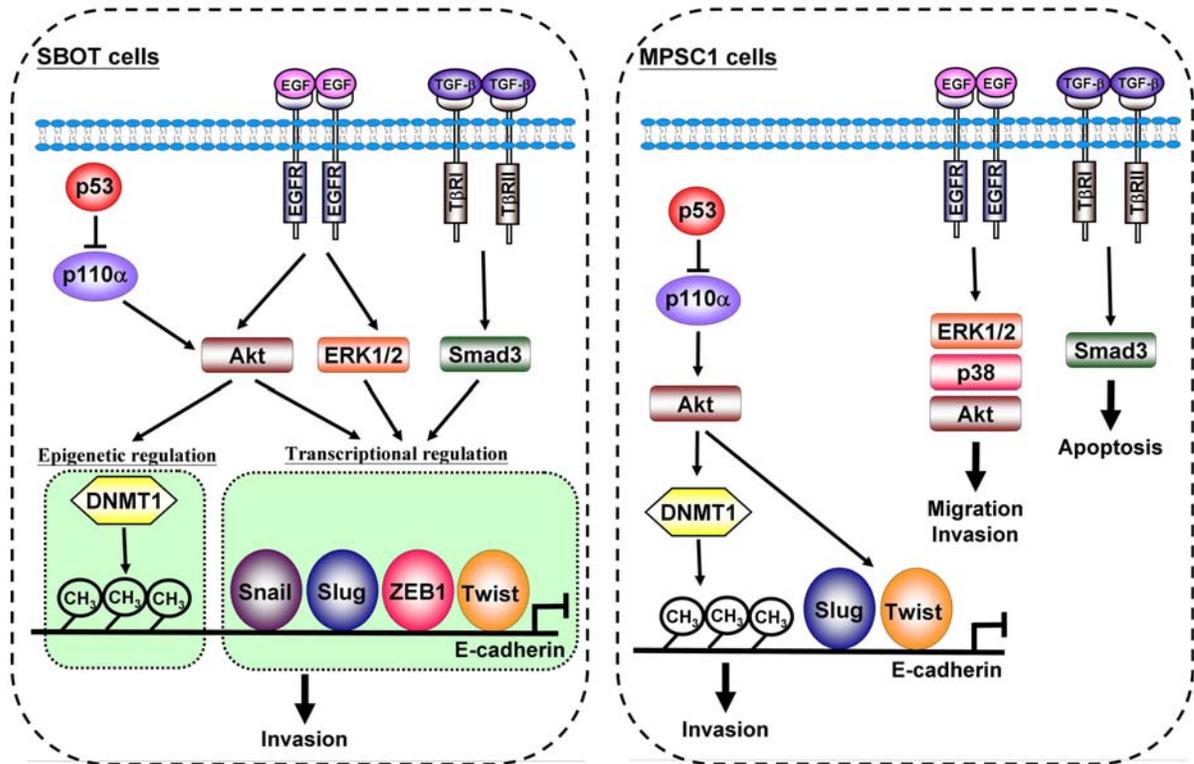
**Figure 7. 1 The summary of the present study**

In SBOT cells (left panel), p53 inhibition increases the expression of p110α (*PIK3CA*) expression and Akt activation. The activated PI3K/Akt signaling pathway mediates the inhibition of p53-induced down-regulation of E-cadherin and cell invasion by transcriptional and epigenetic machineries. In the transcriptional pathway, PI3K/Akt-mediated up-regulation of the E-cadherin transcriptional repressors Slug and Twist contributes to the down-regulation of E-cadherin and, subsequently, to cell invasion. In the epigenetic pathway, PI3K/Akt-mediated up-regulation of DNMT1 enhances E-cadherin promoter methylation, which down-regulates E-cadherin and contributes to cell invasion. Treatment with EGF and TGF-β induces SBOT cell invasion by down-regulating E-cadherin through ERK1/2-, Akt- or Smad3-mediated up-regulation of the E-cadherin transcriptional repressors Snail, Slug, Twist and ZEB1. Conversely, in LGSC-derived MPSC1 cells (right panel), enhancing the levels of p53 up-regulates E-cadherin and decreases cell invasion by diminishing the PI3K/Akt-mediated down-regulation of Slug, Twist and DNMT1. Treatment with EGF induces cell migration and invasion by an E-cadherin-independent pathway, while treatment with TGF-β induces cell apoptosis.
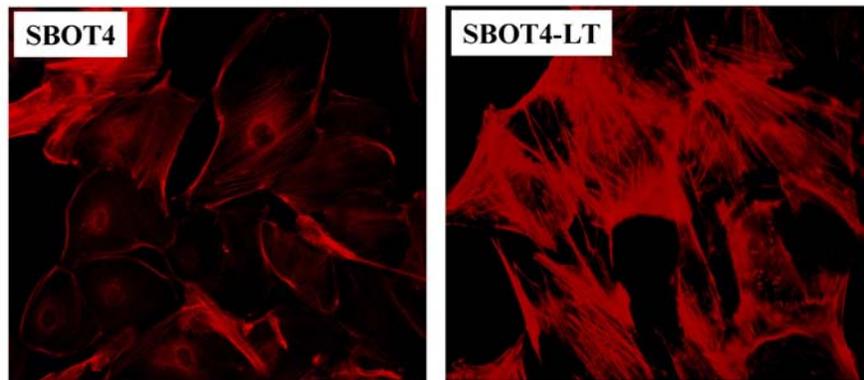
**Figure 7. 2 Expression of actin stress fibers in SBOT4 and SBOT4-LT cells**

Cells were seeded on the cover slips, and the expression of actin stress fibers was examined by rhodamine-conjugated phalloidin staining.
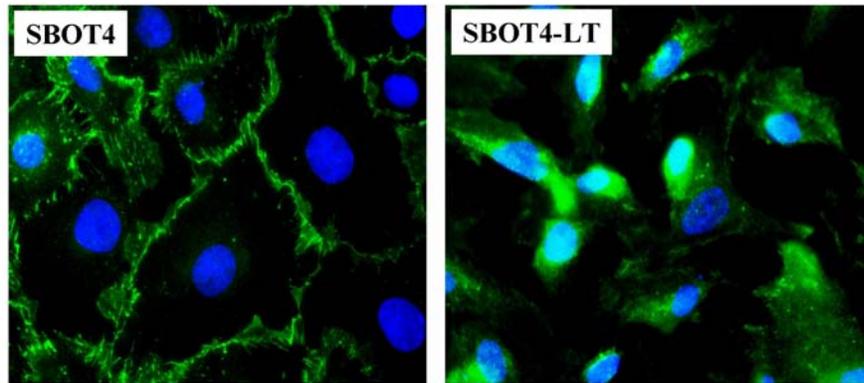
**Figure 7. 3 Expression and localization of β-catenin in SBOT4 and SBOT4-LT cells**

Cells were seeded on the cover slips, and the expression and localization of β-catenin was examined by immunofluorescence staining. The nuclei were stained with Hoechst 33258.
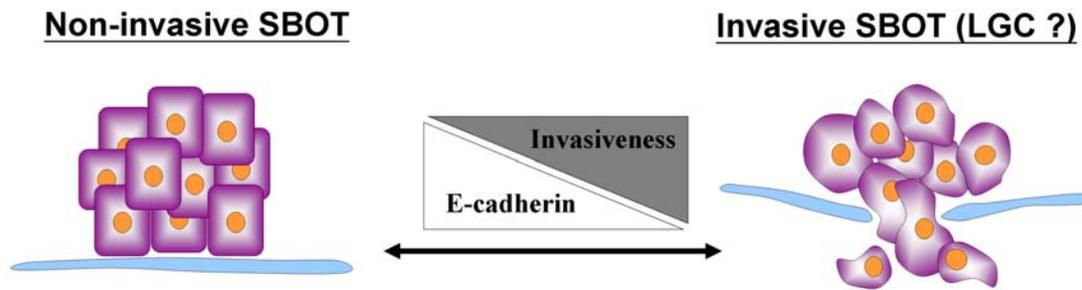
**Figure 7. 4 Conclusion of the present study**

E-cadherin functions as a tumor suppressor in serous borderline ovarian tumor by inhibiting cell invasion. Down-regulation of E-cadherin in serous borderline ovarian tumors (SBOT) may be a critical step that enhances the transition to invasive low-grade serous carcinomas (LGSC).

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# Appendix

## AWARDS

1. Trainee Research Award for Outstanding Achievement by a Doctoral Student, Child & Family Research Institute, 2012
2. BC Foundation for Non-Animal Research-Evelyn Martin Memorial Fellowship, 2011~2012
3. Predoctoral Travel Award, The 50th Annual Meeting of American Society of Cell Biology, 2010
4. PhD Student Poster Award, CIHR-IHDCYH, Scientific Forum on Global Health, 2010
5. Trainee Travel Award, The 5th Canadian Conference on Ovarian Cancer Research, 2010
6. Four Year Fellowships for PhD Students, University of British Columbia, 2009~2010
7. Trainee Travel Award, The 4th Canadian Conference on Ovarian Cancer Research, 2008
8. University Graduate Fellowships, University of British Columbia, 2007~2008
9. Graduate Studentships, Interdisciplinary Women's Reproductive Health Training Program (funded by the Canadian Institutes of Health Research, CIHR), 2007~2008

## PUBLICATIONS

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2. **Cheng JC**, Auersperg N, Leung PC. (2012) TGF-beta induces serous borderline ovarian tumor cell invasion by activating EMT but triggers apoptosis in low-grade serous ovarian carcinomas cells. *PLoS One.* 7: e42436.

3. **Cheng JC**, Chang HM, Leung PC. (2012) Epidermal growth factor-induced human oviductal epithelial cell invasion by down-regulating E-cadherin expression. *J Clin Endocrinol Metab.* 97: E1380-E1389.

4. **Cheng JC**, Chang HM, Leung PC. (2012) Egr-1 mediates epidermal growth factor-induced downregulation of E-cadherin expression via Slug in human ovarian cancer cells. *Oncogene.* (In press)

5. **Cheng JC**, Auersperg N, Leung PC. (2012) EGF-induced EMT and invasiveness in serous borderline ovarian tumor cells: a possible step in the transition to low-grade serous carcinoma cells? *PLoS One.* 7: e34071.

6.  **Cheng JC**, Chang HM, Leung PC. (2011) Wild-type p53 attenuates cancer cell motility by inducing growth differentiation factor-15 expression. ***Endocrinology.*** 152: 2987-2995.

7.  **Cheng JC**, Auersperg N, Leung PC. (2011) Inhibition of p53 represses E-cadherin expression by increasing DNA methyltransferase 1 and promoter methylation in serous borderline ovarian tumor cells. ***Oncogene.*** 30: 3930-3942.

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11. **Cheng JC**, Klausen C, Leung PC. (2010) Hydrogen peroxide mediates epidermal growth factor-induced down-regulation of E-cadherin expression via p38 mitogen-activated protein kinase and Snail in human ovarian cancer cells. ***Mol Endocrinol.*** 24: 1569-1580.

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14. So WK, **Cheng JC**, Poon SL, Leung PC. (2008) Gonadotropin-releasing hormone and ovarian cancer: a functional and mechanistic overview. ***FEBS J.*** 275: 5496-5511.

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**CONFERENCE PROCEEDINGS**

1. **Cheng JC**, Klausen C, Leung PC. (2012) Overexpression of wild-type FOXL2 but not C134W mutant FOXL2 enhances GnRH-induced cell apoptosis by increasing GnRH receptor expression in human granulosa tumor cells. *Academic Day, Department of Obstetrics and Gynaecology, University of British Columbia, Vancouver, Canada.*

2. **Cheng JC**, Klausen C, Leung PC. (2011) Overexpression of wild-type FOXL2 but not C134W mutant FOXL2 enhances GnRH-induced cell apoptosis by increasing GnRH receptor expression in human granulosa tumor cells. *The 44th Annual Meeting of the Society for the Study of Reproduction, Portland, USA.*

3. **Cheng JC**, Klausen C, Leung PC. (2010) Hypoxia-inducible factors-1 mediates epidermal growth factor-induced down-regulation of E-cadherin expression in human ovarian cancer cells. *The 50th Annual Meeting of American Society of Cell Biology, Philadelphia, USA.*

4. **Cheng JC**, Auersperg N, Leung PC. (2010) Inhibition of p53 represses E-cadherin expression by increasing DNA methyltransferase 1 and promoter methylation in serous borderline ovarian tumor cells. *The 5th Canadian Conference on Ovarian Cancer Research, Toronto, Canada.*

5. **Cheng JC**, Auersperg N, Leung PC. (2010) *Inhibition of p53 represses E-cadherin expression by increasing DNA methyltransferase 1 and promoter methylation in serous borderline ovarian tumor cells. Canadian Institutes of Health Research-Institute of Human Development, Child and Youth Health, Scientific Forum on Global Health, Vancouver, Canada.*

6. **Cheng JC**, Auersperg N, Leung PC. (2009) p53 inhibition induces epithelio-mesenchymal transition and increases invasiveness of serous borderline ovarian tumor cells. *Academic Day, Department of Obstetrics and Gynaecology, University of British Columbia, Vancouver, Canada.*

7. **Cheng JC**, Woo MM, Salamanca CM, Leung PC, Auersperg N. (2008) p53 inhibition

induces epithelio-mesenchymal transition and increases invasiveness of serous borderline ovarian tumor cells. *The 4th Canadian Conference on Ovarian Cancer Research, Montreal, Canada.*

8.  Wu HM, **Cheng JC**, Wang HS, Lai CH, Leung PC. (2008) Activation of extracellular signal-regulated protein kinase and p38 mitogen-activated protein kinase in type II gonadotropin-releasing hormone-induced cell growth inhibition and apoptosis of the human endometrial cancer cells. *The 99th Annual Meeting of American Association for Cancer Research, San Diego, USA.*

9.  **Cheng JC**, Woo MM, Salamanca CM, Leung PC, Auersperg N. (2008) Inhibition of the tumor suppressor p53 induces invasiveness in serous borderline ovarian tumor cells. *The 99th Annual Meeting of American Association for Cancer Research, San Diego, USA.*