

**THE ROLE OF ACTIVIN B AND TGF β 1 IN REGULATING ENDOMETRIAL CANCER
CELL ADHESION AND MIGRATION**

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES
(Reproductive and Developmental Sciences)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

July 2016

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Abstract

Endometrial cancer is the fourth most common female cancer and the most common gynecological malignancy. Although it comprises only ~10% of all endometrial cancers, the serous histological subtype accounts for ~40% of deaths due to its aggressive behavior and propensity to metastasize. Moreover, the number of endometrial cancer related deaths keeps rising, which can be attributed to the increased incidence of advanced-stage tumor and high risk histologies. Histopathological studies suggest that in non-endometrioid endometrial cancers (type II, mostly serous), elevated expression of activin/inhibin β B subunit is associated with reduced survival and TGF β signalings are closely associated with the neoplastic transformation of human endometrium and the initiation of invasion of endometrial cancer. However, little is known about the specific roles and mechanisms of activin B (β B dimer) and TGF β 1 in type II endometrial cancer cell progression. We hypothesized that integrin α v β 3, E-cadherin, and PTEN play critical roles in activin B or TGF β 1 induced endometria cancer cell adhesion or migration. Type II endometrial cancer cell lines KLE, HEC-1B and HEC-50 were used as study models. Cancer cell adhesion was assessed by extracellular matrix coated 96 well adhesion assays. Cancer cell migration or invasiveness was assessed by transwell assays without or with coated matrigel following exposure to recombinant human activin B or TGF β 1. Small interfering RNA (siRNA)-mediated knockdown or vector-mediated overexpression approaches were used to investigate the molecular determinants of activin B or TGF β 1-mediated functions. In summary, our results demonstrate that SMAD-mediated integrin β 3 up-regulation by activin B promotes type II endometrial cancer cell adhesion and migration while ERK1/2-SNAIL-mediated E-cadherin down-regulation by activin B plays important roles in cancer migration. Moreover, TGF β 1

induces type II endometrial cancer cell migration via ERK1/2 mediated-up-regulation of integrin $\alpha v \beta 3$. TGF β 1 also promotes cancer cell migration by down-regulating PTEN via both SMAD-dependent and independent pathways. Our findings provide important insights into the molecular mechanisms underlying the effects of activin and TGF β on endometrial cancer cell migration and suggest novel therapeutic targets for treating type II endometrial cancer.

Preface

This study was approved by the University of British Columbia Research Ethics Board.

Certificate Number: H07-01149.

A version of chapter 3 has been published

Siyuan Xiong, Christian Klausen, Jung-Chien Cheng, Hua Zhu, Peter C.K. Leung. (2015) Activin B induces human endometrial cancer cell adhesion, migration and invasion by up-regulating integrin $\beta 3$ via SMAD2/3 signaling. (Oncotarget. 2015 Oct 13;6(31):31659-73)

I was responsible for experiments design, performance and research data analysis. I wrote the manuscript, which was revised by Dr. Christian Klausenr Jung-Chien Cheng and my supervisor Dr. Peter C.K. Leung.

A version of chapter 4 has been published

Siyuan Xiong, Christian Klausen, Jung-Chien Cheng, Jianfang Zhao, Peter C.K. Leung. (2016) Activin B promotes endometrial cancer cell migration by down-regulating E-cadherin via SMAD-independent MEK-ERK1/2-SNAIL signaling. (Oncotarget. 2016 May 19. doi: 10.18632/oncotarget.9483.)

I designed and conducted all the testing as well as statistical analysis. I wrote the manuscript, which was further refined by Dr. Christian Klausen and my supervisor Dr. Peter C.K. Leung.

A version of chapter 5 is in revision

Siyuan Xiong, Christian Klausen, Peter C.K. Leung. TGF β 1 induces human endometrial cancer cell adhesion and migration by up-regulating integrin β 3 and α v via SMAD-independent MEK-ERK1/2 signaling.

I was responsible for experiments design, performance and research data analysis. I wrote the manuscript, which was revised by Dr. Christian Klausen and my supervisor Dr. Peter C.K. Leung.

A version of chapter 6 is ready to be submitted

Siyuan Xiong, Jung-Chien Cheng, Christian Klausen, Jianfang Zhao, Peter C.K. Leung. TGF β 1 stimulates migration of type II endometrial cancer cells by down-regulating PTEN via activation of SMAD and ERK1/2 signaling pathways.

I was responsible for experiments design, performance and research data analysis. I wrote the manuscript, which was revised by Dr Jung-Chien Cheng and my supervisor Dr. Peter C.K. Leung.

Table of contents

| | |
|---|-------------|
| Abstract..... | ii |
| Preface..... | iv |
| Table of contents..... | vi |
| List of tables..... | ix |
| List of figures..... | x |
| List of abbreviations | xiii |
| Acknowledgements | xvi |
| Chapter 1: Introduction | 1 |
| 1.1 Cancer | 1 |
| 1.2 Endometrium..... | 2 |
| 1.3 TGF β superfamily in endometrium | 10 |
| 1.4 Integrins | 17 |
| 1.5 Cadherins | 19 |
| 1.6 PTEN..... | 21 |
| Chapter 2: Rationale and objectives | 24 |
| 2.1 Rationale | 24 |
| 2.2 Overall hypothesis | 27 |
| 2.3 Aim of the study..... | 28 |
| 2.4 Study models..... | 28 |
| 2.5 The specific objectives of this study | 28 |
| Chapter 3: Activin B induces human endometrial cancer cell adhesion, migration and invasion by up-regulating integrin β3 via SMAD2/3 signaling..... | 31 |

| | | |
|--|-----------------------------|------------|
| 3.1 | Introduction..... | 31 |
| 3.2 | Results..... | 33 |
| 3.3 | Discussion..... | 37 |
| 3.4 | Materials and methods | 42 |
| Chapter 4: Activin B promotes endometrial cancer cell migration by down-regulating E-cadherin via SMAD-independent MEK-ERK1/2-SNAIL signaling | | 59 |
| 4.1 | Introduction..... | 59 |
| 4.2 | Results..... | 61 |
| 4.3 | Discussion..... | 65 |
| 4.4 | Materials and methods | 69 |
| Chapter 5: TGFβ1 induces human endometrial cancer cell adhesion and migration by up-regulating integrin β3 and αv via SMAD-independent MEK-ERK1/2 signaling | | 93 |
| 5.1 | Introduction..... | 93 |
| 5.2 | Results..... | 95 |
| 5.3 | Discussion..... | 99 |
| 5.4 | Materials and methods | 103 |
| Chapter 6: TGFβ1 stimulates type II endometrial cancer cell migration by down-regulating PTEN through SMAD and ERK1/2 signaling pathways | | 123 |
| 6.1 | Introduction..... | 123 |
| 6.2 | Results..... | 125 |
| 6.3 | Discussion..... | 128 |
| 6.4 | Materials and methods | 131 |
| Chapter 7: Conclusion..... | | 145 |

| | | |
|---------------------------|--|------------|
| 7.1 | Conclusion | 145 |
| 7.2 | Overall discussion of this study | 148 |
| 7.3 | Limitations of this study | 154 |
| 7.4 | Future directions | 154 |
| 7.5 | Significance and translational potential | 155 |
| Bibliography | | 158 |
| Appendix..... | | 174 |

List of tables

| | |
|---|----|
| Table 1. Endometrial cancer FIGO grade | 6 |
| Table 2. TGF β super family ligands and receptors | 16 |

List of figures

| | |
|---|----|
| Figure 1 Endometrial cancer stage from I to IV. | 7 |
| Figure 2 Activin and inhibin homodimers showing different combinations of protein subunits.... | 11 |
| Figure 3 Activin and TGF β signaling pathways. | 17 |
| Figure 3. 1 Elevated inhibin β B is associated with reduced disease free survival and a trend towards reduced overall survival in serous endometrial cancers..... | 47 |
| Figure 3. 2 Activin B increases endometrial cancer cell migration, invasion and adhesion..... | 48 |
| Figure 3. 3 SB431542 abolishes activin B-induced cell migration, invasion and adhesion. | 49 |
| Figure 3. 4 Activin B up-regulates integrin β 3 expression in endometrial cancer cells. | 50 |
| Figure 3. 5 Effects of activin B on SMAD2 and SMAD3 phosphorylation in endometrial cancer cells. | 51 |
| Figure 3. 6 SMAD4 is required for the up-regulation of integrin β 3 by activin B. | 52 |
| Figure 3. 7 SMAD2 and SMAD3 are required for the up-regulation of integrin β 3 by activin B. | 54 |
| Figure 3. 8 Integrin β 3 mediates activin B-induced endometrial cancer cell migration, invasion and adhesion to vitronectin. | 56 |
| Figure 3. 9 A schematic illustration of Chapter 3 data | 58 |
| Figure 4. 1 Enhanced activin B signaling may contribute to the down-regulation of E-cadherin in serous endometrial cancers..... | 74 |
| Figure 4. 2 Enhanced activin B signaling is not associated with the down-regulation of E-cadherin in endometrioid endometrial cancers. | 76 |

| | |
|---|-----|
| Figure 4. 3 Activin B down-regulates E-cadherin expression in human endometrial cancer cells. | 78 |
| Figure 4. 4 SMAD4 is not required for the down-regulation of E-cadherin by activin B. | 79 |
| Figure 4. 5 SMAD2 and SMAD3 are not required for activin B-induced down-regulation of E-cadherin. | 80 |
| Figure 4. 6 MEK-ERK1/2 signaling is required for the down-regulation of E-cadherin by activin B. | 82 |
| Figure 4. 7 Effects of activin B on AKT and p38 MAPK phosphorylation in endometrial cancer cells. | 84 |
| Figure 4. 8 Activin B up-regulates SNAIL via MEK-ERK1/2 signaling. | 85 |
| Figure 4. 9 Activin B does not alter the mRNA levels of SLUG, TWIST and ZEB1. | 86 |
| Figure 4. 10 SNAIL is required for the down-regulation of E-cadherin by activin B. | 87 |
| Figure 4. 11 MEK-ERK1/2 signaling is required for activin B-induced cell migration. | 89 |
| Figure 4. 12 Forced-expression of E-cadherin inhibits activin B-induced cell migration. | 91 |
| Figure 4. 13 A schematic illustration of Chapter 4 data | 92 |
| Figure 5. 1 TGF β 1 increases endometrial cancer cell adhesion and migration. | 108 |
| Figure 5. 2 TGF β 1 up-regulates integrin β 3 and α v expression in endometrial cancer cells. | 109 |
| Figure 5. 3 Enhanced TGF β signaling may contribute to the up-regulation of integrin β 3 and α v in serous endometrial cancers. | 111 |
| Figure 5. 4 SMAD2 and SMAD3 are not required for TGF β 1-induced up-regulation of integrin β 3 and α v. | 113 |
| Figure 5. 5 MEK-ERK1/2 signaling is required for the up-regulation of integrin β 3 and α v. | 115 |

| | |
|---|-----|
| Figure 5. 6 MEK-ERK1/2 signaling is required for TGFβ1-induced cell adhesion to vitronectin and migration. | 117 |
| Figure 5. 7 Integrin β3 and αv mediate TGFβ1-induced endometrial cancer cell adhesion to vitronectin. | 118 |
| Figure 5. 8 Integrin β3 and αv mediate TGFβ-induced endometrial cancer cell migration. | 120 |
| Figure 5. 9 A schematic illustration of Chapter 5 data | 122 |
| Figure 6. 1 TGFβ1 stimulates type II endometrial cancer cell migration. | 136 |
| Figure 6. 2 TGFβ1 down-regulates PTEN expression in type II endometrial cancer cells. | 137 |
| Figure 6. 3 Overexpression of PTEN abolishes TGFβ1-stimulated cell migration. | 138 |
| Figure 6. 4 Inhibition of AKT signaling attenuates TGFβ1-stimulated cell migration. | 139 |
| Figure 6. 5 Activation of SMAD signaling is required for TGFβ1-induced down-regulation of PTEN expression. | 140 |
| Figure 6. 6 Activation of ERK1/2 signaling is required for TGFβ1-induced down-regulation of PTEN expression. | 142 |
| Figure 6. 7 A schematic illustration of Chapter 6 data. | 144 |
| Figure 7. 1 A schematic diagram of the proposed mechanisms of activating B and TGFβ1 regulating endometrial cancer cell adhesion, migration and invasion. | 148 |
| Figure 7. 2 Elevated inhibin βB is not associated with reduced disease free survival and a trend towards reduced overall survival in endometrioid endometrial cancers. | 156 |

List of abbreviations

| | |
|-------|--|
| ACVR | Activin receptor |
| AKT | Protein kinase B |
| ALK | Activin receptor-like kinase |
| ANOVA | Analysis of variance |
| BSA | Bovine serum albumin |
| °C | Degrees Celsius |
| cDNA | Complementary DNA |
| DMEM | Dulbeco's modified eagle's medium |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxynucleoside triphosphate |
| ECL | Enhanced chemiluminescence |
| ECM | Extracellular matrix |
| ELISA | Enzyme-linked immunosorbent assay |
| EMT | Epithelial mesenchymal transition |
| ERK | Extracellular signal-regulated kinase |
| FAK | Focal Adhesion Kinase |
| FBS | Fetal bovine serum |
| FGFR | Fibroblast growth factor receptor |
| FIGO | International Federation of Gynecology and Obsterics |
| G | Grade |
| g | Gram |

| | |
|---------|--|
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| h | Hour |
| kDa | Kilodalton |
| min | Minutes |
| ml | Milliliter |
| MAPK | Mitogen-activated protein kinase |
| MEK | Mitogen-activated protein kinase kinase |
| MMP | Matrix metalloproteinase |
| mRNA | Messenger RNA |
| MSI | Microsatellite-unstable |
| mTOR | Mechanistic target of rapamycin |
| MTT | 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| μL | Microliter |
| nM | Nanomolar |
| PAGE | Polyacrylamide gel electrophoresis |
| PBS | Phosphate buffered saline |
| PI3K | Phosphoinositide 3-kinase |
| PTEN | Phosphatase and tensin homolog |
| RGD | Arg-Gly-Aspartine |
| RNA | Ribonucleic acid |
| RPL | Recurrent pregnancy loss |
| R-SMAD | Receptor-regulated SMAD |
| RT-qPCR | Reverse transcription quantitative polymerase chain reaction |

| | |
|--------------|---|
| SD | Standard deviation |
| SDS | Sodium dodecyl sulfate |
| SEM | Standard error of the mean |
| siRNA | Small interfering RNA |
| SMAD | Sma- and Mad-related protein |
| TCGA | The Cancer Genome Atlas Network |
| TGF- β | Transforming growth factor-beta |
| TIMP | Tissue inhibitor of metalloproteinases |
| TBS | Tris-buffered saline |
| TP53 | Tumor protein p53 |
| Tris-HCl | Tris (hydroxyl methyl)-aminomethane-hydrochloric acid |
| VEGF | Vascular endothelial growth factor |
| ZEB | Zinc finger-E-box-binding homeobox |

Acknowledgements

First of all, I would like to express my deepest gratitude to my supervisor, Dr. Peter C.K. Leung for offering me the opportunity to pursue my Ph.D under his supervision. His understanding, encouragement, support and enthusiasm helped me overcome research difficulties during my study and set an admirable model for my future career.

I offer my enduring gratitude to my supervisory committee, Dr. Mohamed Bedaiwy, Dr. Alexander Beristain, Dr. Christian Klausen and Dr. Dan Rurak, for their invaluable scientific guidance, continuous help and precious comments that enlarged my vision of science and inspired me to conduct research in this field.

My sincere thanks also go to Dr. Christian Klausen for his great advice, support and comments. I am grateful to Ms. Roshni Nair for her excellent administration and consistent support. I would like to express my special thanks to Dr Jung-Chien Cheng and Dr. Hua Zhu who granted their endless help to me from all aspects. All my labmates, we had such a nice study period together and I will definitely miss you all.

I would like to thank my parents for their support, encouragement and understanding throughout my years of education.

Chapter 1: Introduction

1.1 Cancer

Cancer is the general name for a group of diseases with abnormal cell growth and metastasis tendency. Cancerous tumors can invade into adjacent tissues, enter into the circulation system and spread into distant organs while benign tumors don't migrate. Cancer is characterized by six biological capabilities gradually acquired during tumor progression which include sustaining proliferative signaling, evading growth suppressors, anti-apoptosis, limitless replicative potential, inducing angiogenesis, and activating invasion and metastasis [1].

1.1.1 Cancer metastasis and spread

Metastatic cancer is the type of cancer that spread from its original site to other parts of the body. A critical capability of tumor cells that distinguishes them from benign to malignant tumors is invasion and metastasis, which accounts for 90% of cancer related deaths [2]. EMT (Epithelial to mesenchymal transition) has been found to play a critical role in metastasis and is a hallmark in the malignant progression to aggressive carcinoma. Molecularly, EMT is characterized by loss of epithelial characteristics and acquirement of mesenchymal gene expression program such as the down-regulation of E-cadherin and the up-regulation of N-cadherin [3]. Apart from EMT, other genetic and physiological conditions also contribute to metastasis including alterations of extracellular matrix, integrins, cadherins, cell-cell adhesion molecules, metastasis genes and metastasis suppressors [2].

1.2 Endometrium

Endometrium is the mucosa of the uterus. Morphologically it can be divided into an upper two-thirds of “functionalis” layer and a lower one third “basalis” layer. The functional layer is unstable and experiences morphological and functional changes affected by the cyclic release of sexual hormones. It’s the site of proliferation, secretion and degeneration. The basalis layer doesn’t respond to hormone changes and provides the regenerative endometrium following the menstrual loss of the functionalis. The menstrual cycle is the natural cyclic changes that occur in the uterus and ovary in preparation for pregnancy [4]. For the endometrium, the menstrual cycle is divided into the menstruation phase, the proliferative phase and the secretory phase. When implantation doesn’t occur, the levels of circulating estradiol and progesterone hormones decrease with the regression of corpus luteum, which leads to the inflammatory cell influx and production of cytokines, prostaglandins and proteases in the endometrium. These finally result in break-down and shedding of its functional layer. Simultaneously, endometrial repair occurs involving infiltrating leukocytes and their locally secreted factors [5]. The proliferative phase is associated with ovarian follicle growth and increased estrogen secretion. During this phase, the epithelial cells in the glands, endometrial cavity, stromal cells, and endothelial cells proliferate and spiral vessels extend to form a loose capillary network due to the growth stimulation effects of estrogen. After ovulation, the endometrium enters into secretory phase and responds to combined actions of estrogen and progesterone. The progesterone not only induces the differentiation of all compartments within endometrium but also inhibits the proliferation of epithelial cells due to its antagonizing effects on estrogen [6].

1.2.1 Endometrial Cancer

Endometrial cancer is the most common, and second most lethal, gynecological malignancy and the fourth most common female cancer in North America [7]. The number of endometrial cancer related death is steadily increasing [8] [10]. This can be attributed to the increased incidence of advanced-stage tumor and high risk histologies, which are characterized by invasion and metastasis [9]. Although most endometrial cancer occurs in postmenopausal period, 14% of cases are diagnosed in premenopausal women, with 5% of them being younger than 40 years old [10]. The majority of endometrial cancer patients (75%) are diagnosed in the early stages (International Federation of Gynecology and Obstetrics [FIGO] stages I or II), with a five year survival rate at 74-91%. Around 25% women are diagnosed at advanced stage, with a 5 year overall survival at 20-26% in stage IV [7].

1.2.2 Classification systems

Traditionally, based on clinical and epidemiological characteristics, endometrial cancer has been classified into type I and type II defined by Bokhman. Type I tumors are associated with unopposed estrogen stimulation, positive hormone receptor, moderate or high differentiation, rare metastases and favorable outcomes; type II tumors are not hormone dependent, arising in atrophic endometrium, poorly differentiated, frequently metastasized and with poor outcomes [7]. Based on histopathology, endometrial cancer can also be classified into endometrioid (75%), serous (5-10%) and clear cell (1-5%) subtypes. Low grade endometrioid cancer is often associated with endometrial hyperplasia and remains clinically indolent while serous cancer always shows aggressive behavior with unfavorable outcomes. Correlations have been noted between the subtypes in these two classification systems. Type I cancer consists of low grade

endometrioid cancer and type II cancer is mostly non-endometrioid cancer (ie, serous and clear cell cancer). Notably, around 10% to 19% of endometrioid cancer is high grade and has clinical and histopathological features that are similar to type II cancer or intermediate between these two types. At the molecular level, endometrioid (type I) cancer has frequent mutations in PTEN, whereas serous (non-endometrioid, type II) cancer displays recurrent TP53 mutations [7]. Recently, next generation technologies and bioinformatics enable the large-scale genomic sets analysis of endometrial cancer. Thus, based on genome-wide genetic characterization of around 400 endometrial cancer patients, endometrial cancer is classified into four genomic classes: POLE (ultramutated), MSI (microsatellite-instable [hypermuted]), copy number low (endometrioid), and copy number high (serous-like) by TCGA (The Cancer Genome Atlas Network) [11]. The first 3 groups are almost composed exclusively of endometrioid cancer. In contrast, the copy number high group consists mainly of serous and a few high grade endometrioid tumors and therefore has the worst progression free survival rate. Each class has their own characteristic genetic mutations. For examples, the copy number high (serous-like) group has the most extensive copy number alterations and extremely high TP53 mutation frequency (>90%) but low PTEN mutation frequency. By contrast, the remaining 3 groups have high PTEN mutation rate but rare TP53 mutation rate. This integrated genomic analysis provides important molecular insights into tumor classification and may directly guide clinical trials of molecular targeted therapies. More importantly, considering the great genetic and morphological heterogeneity in endometrial cancer, an integrated classification system incorporating clinicopathological and molecular features can help define biologically and clinically relevant subset of endometrial cancer and thus realize the individualized treatment.

1.2.3 Risk factors and protective factors

The risk factors for endometrial cancer are excess exogenous estrogen exposure (unopposed estrogen therapy, tamoxifen), endogenous estrogen (chronic anovulation, obesity, early menarche and late menopause, estrogen secreting tumors), age (The risk of endometrial cancer increases as a woman gets older), family history (Lynch syndrome, BRCA, other genetic syndromes), dietary factors. The protective factors include hormone contraceptives, smoking, increasing age at last birth, diet and exercise, physical activity, coffee, tea and etc) [10, 12].

1.2.4 Clinical presentation and diagnostics

The most common symptom of endometrial cancer is abnormal vaginal bleeding or spotting in women during menopause which occurs in 90% of patients. Patients at advanced stage may display pelvic pain, abdominal distension and weight loss. Endometrial cancer is usually diagnosed by ultrasound, sampling of endometrial tissue such as endometrial biopsy or D&C (dilation and curettage) or hysteroscopy. Preoperative staging maybe required based on the assessment of myometrial and cervical invasion and lymph node metastasis to determine the surgical management. MRI is the best imaging technique for preoperative staging [10].

1.2.5 Tumor grade

Tumor grading depends on the amount of abnormalities of tumors which include the abnormality of tumor cells and tumor tissue organization under a microscope. The grade of endometrial cancer is evaluated by solid tumor growth (the ratio of cancerous tissue to normal tissue) and cancer cell differentiation. Table 1 demonstrates the endometrial cancer FIGO (the International

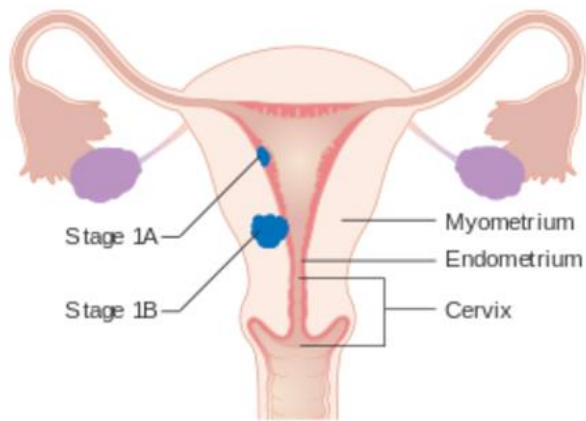
Federation of Gynecology and Obstetrics) grading system (adapted from Canadian Cancer Society, 2016).

| FIGO Grade | Description |
|-------------------|---|
| 1 | 5% or less of tissue comprises solid tumor Well-differentiated |
| 2 | 6%-50% of tissue is solid tumor Moderately differentiated |
| 3 | More than 50% of tissue is solid tumor Poorly differentiated |

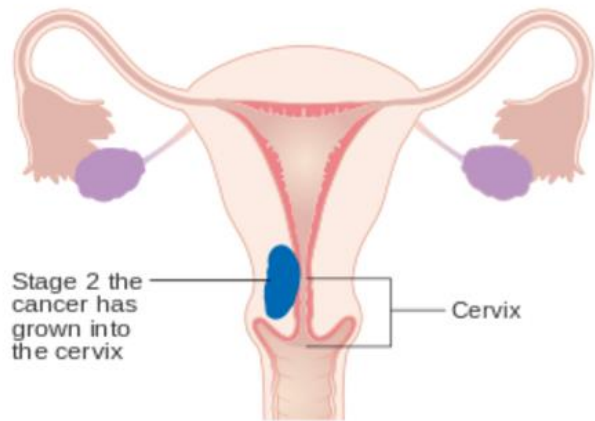
Table 1 Endometrial cancer FIGO grade

1.2.6 Staging

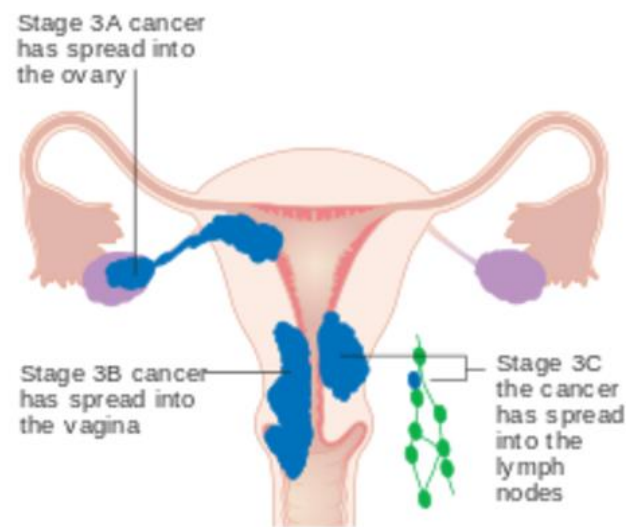
Cancer staging generally takes into account the site of the primary tumor, cell type, tumor size, the extent of regional lymph node involvement, tumor invasion and metastasis into distant organs, and tumor grade (National Cancer Institute, 2015). The major purpose of staging is to know the spreading or metastasis of cancer and to determine the treatment plan. The most commonly used staging system for endometrial cancer is the FIGO system. It is based on surgical staging including assessment of the extent of myometrial invasion and metastasis to local or distant sites [10]. For detailed information, please refer to Figure 1 and “Stage information for Endometrial Cancer” in the National Cancer Institute website [13].



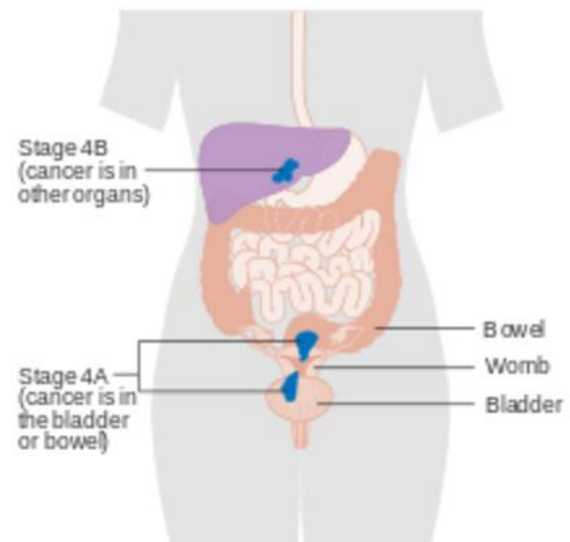
Stage IA and IB endometrial cancer



Stage II endometrial cancer



Stage III endometrial cancer



Stage IV endometrial cancer

Figure 1 Endometrial cancer stage from I to IV. Images are attributed to Cancer Research UK

/ Wikimedia Commons.

1.2.7 Treatment

Surgery is the most-adopted treatment for endometrial cancer consisting of hysterectomy, often along with a salpingo-oophorectomy and removal of lymph nodes. Patients with uterine-confined disease with low-risk features (around 55%) need surgery only. They have a 95% possibility of relapse-free survival at 5 years. For other patients with high risk features or metastasis and recurrence, radiotherapy, hormone therapy, chemotherapy or combinational therapy are also needed. To date, no approved molecular targeted therapies are available for treating endometrial cancer, but many molecular-targeted drugs for endometrial cancer are under clinical trials such as mTOR inhibitors, PI3K inhibitors, MEK inhibitors, ErbB family inhibitors and FGFR/VEGFR inhibitors. The multi-targeted VEGF/FGFR inhibitors (brivanib, lenvatinib) have resulted in encouraging results (response rates 14%-19%) [10].

1.2.8 Models in endometrial cancer research

Immortalized endometrial cancer cell lines or athymic nude mice models transplanted with cancer cells are the mostly adopted models to investigate mechanisms underlying the progression of endometrial cancer and therapeutic regimens [14]. Generally, immortalized endometrial cancer cell lines are also divided into type I and type II subtypes to represent these two types of endometrial cancer, based on their morphological, clinic-pathological, hormone receptor status, and other molecular characteristics [15].

1.2.8.1 Type I endometrial cancer cell lines

Ishikawa H cells were isolated from a moderately-differentiated tumor in the uterus of a patient diagnosed with stage II endometrial cancer. The tumor had migrated into the entire uterine cavity

and half of the uterine wall but without detected metastasis to lymph nodes. Ishikawa H cells have progesterone and estrogen receptors, hormone responsiveness, lost PTEN expression and a doubling time of 36h, making it a good model for type I endometrial cancer [17]. ECC-1 endometrial adenocarcinoma cells originated from a transplantable endometrial cancer called EnCa101. The moderately differentiated endometrial cancer, EnCa101, was first transplanted into nude mice. Then tumors same to the original neoplasm were formed in the mice. ECC-1 cell line was then derived from this tumor and established in monolayer culture [20]. RL95-2 cell line was derived from a Grade 2 moderately differentiated adenosquamous carcinoma of the endometrium. The cells are characteristically epithelioid with well-defined junction complexes and tend to form gland like structures [16, 17].

1.2.8.2 Type II endometrial cancer cell lines

HEC-1 cell line was the first established cell line of human endometrial cancer. The tumor tissues obtained from a 71-year-old female with Grade 2 endometrial carcinoma were placed into culture as an explant plasma clot culture. Then the outgrown cells were sub-cultured for 3 generations to select epithelial cell types. Then the selected epithelial cells were transferred into the monolayer culture. After that the majority of the cultured cells (HEC-1A) showed a steady growth while a minority of cells (HEC-1B) displayed a 2-month stationary period at the 8th to 9th generation [18]. HEC-50 was obtained from the ascitic fluid of a patient with recurrent Grade 3 cancer [19]. HEC-50co, a subline clonally derived from HEC-50, could not form glands in tissue culture or in the animal models and it expresses little or no steroid hormone receptors. It also has lost P53 expression. Moreover, the cells can sub-differentiate into a papillary serous phenotype in the mouse model. All these characteristics indicate HEC-50co cell a good representative of

type II endometrial cancer [15]. KLE is a cell line derived from a poorly differentiated metastatic endometrial carcinoma in a 64-year old patient. Its cytosol contains a specific binder for estradiol, but there is no estrogen receptor in the nucleus and translocation of the estrogen to the nucleus fails to occur [16, 20]. Based on endometrial cancer cell molecular profiling of TCGA, KLE, HEC-1A and HEC-1B cell have mutations in TP53 but none in PTEN, which makes them representative of type II endometrial cancer. The KLE, HEC-1B and HEC-50 cell lines have been used widely as type II endometrial cancer models [15, 16, 21-23]

1.3 TGF β superfamily in endometrium

Although the cyclic alterations of endometrium throughout the menstrual cycle are mainly driven by dynamic change of estrogen and progesterone levels, growth factors and cytokines have been shown to play important roles in the regulation of endometrium growth, differentiation and interaction with steroid hormones. These growth factors and cytokines may come from constituent epithelial, mesenchymal or inflammatory cells. Therefore, endometrium is a complicated interactive system which involves a complex network of intercellular and intracellular signalings formed by different growth factors, cytokines, hormones and cell types to regulate the proliferation, differentiation and the function of endometrium [24, 25]. Among these, the transforming growth factor-beta (TGF β) superfamily members including TGF β 1, TGF β 2, TGF β 3, activin, inhibin, bone morphogenetic protein (BMP), Müllerian inhibiting substance (AMH), and several other proteins are abundantly and dynamically expressed in the endometrium and placenta [5]. The TGF β superfamily members are closely related to tissue remodeling events and reproductive processes during the menstrual cycle and establishment of pregnancy [5].

1.3.1 Activin and TGF β

Activin and inhibin proteins are disulphide-linked heterodimeric or homodimeric proteins that have almost opposite effects but share similar or identical β subunits [26]. Activins are homo or heterodimers of inhibin β subunits. Inhibins are heterodimers of a common α subunit and a β subunit. Depending on the subunits combination, there are three primary isoforms of activins [activin A ($\beta A\beta A$), activin AB ($\beta A\beta B$) and activin B ($\beta B\beta B$)] and two primary isoforms of inhibins [inhibin A ($\alpha\beta A$) and inhibin B ($\alpha\beta B$)]. Three other β subunits (βC , βD and βE) have also been found in human. However, little is known about their biological significance [26]. Three TGF β isoforms (TGF $\beta 1$, TGF $\beta 2$, and TGF $\beta 3$) have been identified in mammals which share about 70% homology [27] and TGF $\beta 1$ is the most abundant and studied isoform. TGF β isoforms are secreted from cells as latent homodimeric polypeptide bound to other extracellular proteins. The mature, bioactive ligand is produced on proteolytic cleavage of the latent complex [28].

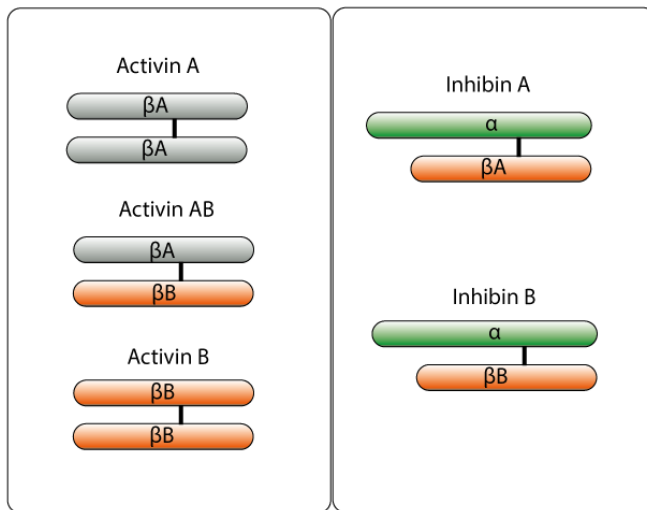


Figure 2 Activin and inhibin homodimers showing different combinations of protein subunits. Images are attributed to Bio-Rad: Inhibin and Activin Mini-review.

1.3.2 TGF β family receptors and signaling pathways

The canonical TGF β signaling pathway involves the activation of a complex of type I and type II transmembrane serine/threonine kinase receptors and the later participation of intracellular effectors, SMADs. TGF β superfamily members first bind to extracellular domain of type II receptors, which in turn activate type I receptors. After that, receptor SMADs (R-SMADs) 1/5/8 or 2/3 are phosphorylated by the type I receptor. A complex of R-SMADs with a common SMAD4 is formed and then translocates into the nucleus to modulate gene expression. There are five type II receptors (TGF β R2, ACVR2A, ACVR2B, BMPR2 and AMHR2) and seven type I receptors (ACVRL1, ACVR1, BMPR1A, ACVR1B, TGF β RI, BMPR1B and ACVR1C), which are also called activin receptor-like kinase (ALK) 1-7, recognized in mammals [29]. TGF β family can also signal via SMAD-independent non-canonical pathways such as the phosphoinositide 3-kinase (PI3K)/AKT, mitogen-activated protein kinase (MAPK) and Rho GTPase pathways [30].

Specifically, activins bind to the extracellular domain of constitutively active activin type II (ACVR2A) or activin type IIB (ACVR2B) receptors while TGF β s bind to TGFBR2. For the type I receptor, activins bind to ActRIB (ALK4) and ActRI (ALK2) with high affinity among seven ALK receptors, whereas TGF β s preferentially signal through ALK5 [31]. Activin AB and Activin B have also been reported to signal through ALK7 [32]. TGF β signaling and activin signaling both operate through canonical SMAD2/3-SMAD4 signaling and non-canonical signalings, such as AKT/PI3K, MAPK/ERK and Wnt/ β -catenin. Despite their overlapping SMAD-dependent and independent pathways, activins and TGF β s signal through common, as

well as different, downstream transcriptional targets, resulting in similar or different functional consequences [31].

1.3.3 SMAD signaling pathways

The SMAD signaling is the most commonly used and therefore classical signaling pathway in TGF β superfamily. SMADs act as the intracellular mediators to directly transduce extracellular stimulus from the cell-surface receptors to the nucleus. There are eight SMADs in most vertebrates which are quite conservative. SMAD family in vertebrates can be divided into three groups which are Receptor-regulated SMADs or R-SMAD (SMAD1, SMAD2, SMAD3, SMAD5, and SMAD8), Common mediator SMAD (Co-SMAD) SMAD4, and Inhibitor SMAD (I-SMAD) SMAD7. R-SMADs can be further subdivided into BMP-activated SMADs (SMAD1, SMAD5, SMAD8) or TGF β -activated SMADs (SMAD2, SMAD3), depending on type I receptor preferences. As for Activins and TGF β s, ligand-type II receptor interaction will trigger the phosphorylation of type I receptor which then phosphorylate SMAD2 or SMAD3. These R-SMADs would then form heterodimers or heterotrimers with SMAD4 which would translocate into the cell nucleus. In nucleus, SMAD complex would cooperate with sequence-specific transcription factors and co-regulators to regulate gene expressions [30].

1.3.4 Activin and TGF β isoforms in endometrium

Inhibin β subunits (β A and β B) are primarily expressed in endometrial glands in the non-pregnant endometrium, with maximal levels seen in the secretory phase. Expression of inhibin α subunit has also been documented in glandular epithelium, but to a lesser degree than inhibin β

subunits, indicating that activin dimers rather than inhibins are preferentially produced. Transcripts encoding inhibin β A and β B subunits as well as activin receptors have been detected in primary cultures of normal endometrial epithelial and stromal cells [9]. At the protein level, secreted activin A has been detected in conditioned medium from normal endometrial epithelial and stromal cells [9].

The level and cell-specific expression of TGF β s within different endometrial regions are affected by the cyclic changes of estrogen and progesterone during the menstrual cycle, with TGF β 2 mainly localizing to stroma while TGF β 1 and TGF β 3 present in both epithelial and stromal cells. TGF β 1 has also been found in endometrial glands and present in uterine fluid. Cyclic changes of TGF β 1 expression are not evident, while maximal glandular production of TGF β 3 occurs in the late secretory phase and up-regulated TGF β 2 in the secretory-phase promotes endometrium differentiation [9, 26].

1.3.5 Activins and TGF β s in endometrial cancer

Early studies demonstrated inhibin β subunit expression, activin secretion and activin receptor expression in neoplastic endometrial tissues and/or endometrial cancer cell lines [33-35]. More importantly, elevated inhibin β B is associated with reduced cause specific survival in non-endometrioid tumors [36]. Though histopathological studies suggest activin B (inhibin β B dimers) may be linked to poor survival in the most lethal subtype of endometrial cancer, few studies have examined the effects of activins on endometrial cancer cells.

As for TGFβs, there is a statistically significant gradual increase in the immune-staining of all three TGFβs progressing from the normal proliferative endometrium to simple hyperplasia and on to complex hyperplasia [37]. Consistently, TGFβ1 plasma level is greater in Stage-Ib and Stage-Ic patients than in Stage-Ia patients [38]. TGFBR2 protein level estimated by ELISA is higher in endometrial cancer compared to normal endometrial tissues [39]. Significantly elevated protein level of TGFBR2 was also noted in endometrial cancer with myometrial invasion compared to those non-infiltrating tumors [39, 40]. Moreover, in vitro experiments showed that TGFβ1 could promote the invasiveness of KLE, HEC-1A and RL95-2 endometrial cancer cells [38, 41]. While much has been written on the expression and roles of TGFβ1 signaling in endometrial cancer, there is little information on the mechanisms of TGFβ1 that governs the migration.

| Receptor type | Ligand | R-SMAD |
|-------------------|--|------------------------|
| Type I receptor | | |
| ALK1/ACVRL1 | TGF- β , BMP9 and BMP10 | SMAD1, SMAD5 and SMAD8 |
| ALK2/ACVR1 | BMPs and GDFs | SMAD1, SMAD5 and SMAD8 |
| ALK3/BMPRI1A | BMPs | SMAD1, SMAD5 and SMAD8 |
| ALK4/ACVR1B | Activins, GDF8/myostatin and GDF11 | SMAD2 and SMAD3 |
| ALK5/TGFBR1 | TGF- β s, GDF/myostatin and GDF11 | SMAD2 and SMAD3 |
| ALK6/BMPRI1B | BMPs | SMAD1, SMAD5 and SMAD8 |
| ALK7/ACVR1C | BMP16/nodal | SMAD2 and SMAD3 |
| Type II receptor | | |
| TGFBR2/TBRII | TGF- β s | |
| BMPRII/BMPRII | BMPs and GDFs | |
| ACVR2/ActRIIA | Activins, BMPs and GDFs | |
| ACVR2B/ActRIIB | Activins, BMPs, GDFs and BMP16/nodal | |
| AMHR2/AMHRII | MIF | |
| Type III receptor | | |
| Betaglycan | TGF- β 1, TGF- β 2, TGF- β 3, activin-A, BMP2, BMP4, BMP7 and GDF5 | |
| Endoglin | TGF- β 1, TGF- β 3, activin-A, BMP2, BMP7 and BMP9 | |

Table 2 TGF β super family ligands and receptors. This table is adapted from Drabsch, Y. & ten Dijke, P. Cancer Metastasis Rev (2012) 31: 553. doi:10.1007/s10555-012-9375-7.

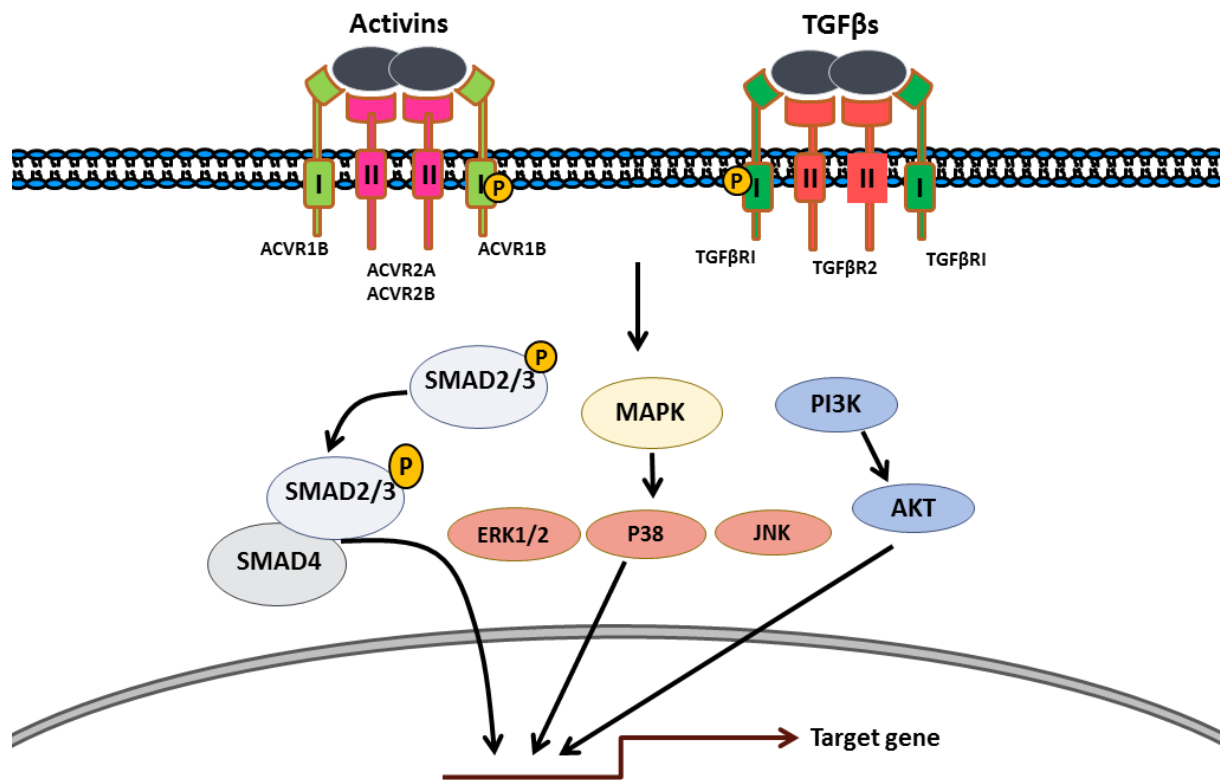


Figure 3 Activin and TGFβ signaling pathways.

1.4 Integrins

Integrins are a diverse family of glycoproteins that form heterodimeric receptors for ECM (extracellular matrix) molecules. They are generally composed of a α subunit and a β subunit non-covalently bound to each other, with each subunit having a large extracellular domain, a single trans-membrane domain, and a short, non-catalytic cytoplasmic domain. There are 18 α -subunits and 8 β -subunits, leading to at least 25 distinct integrins. Each integrin has its own unique set of ligands. For examples, integrin $\alpha\beta3$ recognizes its ECM by a short peptide RGD (Arg-Gly-Aspartine) and binds a wide range of ECM molecules including fibronectin, fibrinogen, vitronectin, and proteolysed forms of collagen and laminin. Integrins participate in regulating

cell adhesion, cell survival, proliferation, migration and invasion. Upon ligand binding, integrins cluster into the focal contacts that contain various actin-associated proteins and link the integrin to the cytoskeleton. Although integrins don't have intrinsic enzymatic and kinase activity, they can activate kinases such as FAK and SRC family to further phosphorylate downstream signalings and regulate cell shape, proliferation and migration [42].

1.4.1 Integrin $\alpha v\beta 3$ in cancer

Tumor cells usually develop dramatic changes in levels of integrin expression and integrin affinity for ECM substrates. The notable differences in cell surface expression and distribution of integrins in malignant tumors compared with pre-neoplastic tumors of the same type have been documented in numerous cancers [43]. $\alpha v\beta 3$ integrin is widely expressed on blood vessels of human tumors and tumor cells, and closely associated with tumor progression [44]. For examples, $\alpha v\beta 3$ is strongly expressed at the invasive front of malignant melanoma cells and angiogenic blood vessels, but weakly expressed in pre-neoplastic melanomas and quiescent blood vessels [45]. Overexpression of $\alpha v\beta 3$ integrin is associated with bone metastasis and induces tumor invasion in breast cancer. Expression of $\alpha v\beta 3$ also results in metastasis to bone in prostate carcinoma cells [46].

1.4.2 Expression of integrin $\alpha v\beta 3$ in endometrium

Integrin $\beta 3$ subunit is a well-known marker of endometrial receptivity [47]. The expression of αv and $\beta 3$ undergoes cycle specific changes in endometrium. Immunostaining for αv increases throughout the menstrual cycle while expression of $\beta 3$ subunit suddenly appears in the glandular epithelium around implantation window. Moreover, mid-secretory phase biopsies from woman

with unexplained RPL (recurrent pregnancy loss) show significantly reduced integrin $\beta 3$ expression [45]. The unique expression of integrin $\beta 3$ subunit in endometrium during implantation period indicates its role in implantation [48, 49]. Integrin $\alpha v\beta 3$ is also expressed in endometrioid and serous endometrial cancers [50, 51]. However, its clinical and/or therapeutic relevance in these tumors has yet to be defined.

1.5 Cadherins

Cadherins are transmembrane glycoproteins that mediate calcium-dependent cell-cell adhesion [52]. The cadherin family can be subdivided into classical cadherins (type I and type II cadherins), desmosomal cadherins, and seven-pass transmembrane or flamingo cadherins according to their molecular characteristics [53]. All classical cadherin members are composed of an extracellular domain that includes one or more cadherin repeats, and transmembrane and cytoplasmic domains. Both type I and type II cadherins have five extracellular repeats. Type I are different from type II in that they have a histidine, alanine, valine (HAV) tripeptide within the most N-terminal extracellular repeat (EC1), which is essential for homotypic interaction of the cadherins. Type I and type II cadherins are transmembrane components of specialized cell junction regions called adherens junctions [54]. Their cytoplasmic domains interact with several adaptor proteins such as catenins and vinculin, which link the cadherin to the actin cytoskeleton and facilitate the clustering into the junctional structure [54]. E-cadherin (epithelial-cadherin, CDH1), N-cadherin (neural cadherin, CDH2), P-cadherin (placental cadherin, CDH3), R-cadherin (retinal cadherin, CDH4) and M-cadherin (mytubule cadherin, CDH15) belong to type I classical cadherins, while VE-cadherin (vascular endothelial cadherin, CDH5) and OB-cadherin

(osteoblast cadherin, CDH11) belong to type II classical cadherins. Classical cadherins are important for normal development, and alterations in cadherin function and expression have been implicated in tumorigenesis [55, 56].

1.5.1 E-cadherin in cancer

Loss of E-cadherin expression at protein and/or transcriptional levels is the hallmark of EMT, both in embryonic development and cancer. E-cadherin is a calcium dependent trans-membrane glycoprotein that plays a key role in the formation of adherens junctions between epithelial cells. During the cancer progression, E-cadherin can be functionally inactivated or silenced by different mechanisms involving somatic mutations, promoter hypermethylation, histone deacetylation, post-translational control and, most frequently, transcriptional repression. Several EMT inducing transcriptional regulators such as SNAIL, SLUG, TWIST and ZEB1 repress E-cadherin transcription through interaction with E-boxes at the E-cadherin promoter region [57]. Reduced E-cadherin expression or loss of function is correlated with metastasis and adverse clinical outcomes in many types of cancer [58-62], whereas its overexpression markedly impairs cancer cell invasiveness [59, 60, 63, 64].

1.5.2 Expression and function of E-cadherin in endometrium

In endometrial cancer, reduced E-cadherin expression is associated with advanced stage [65, 66], poor differentiation [65, 67], deep myometrial invasion [66-68], lymph node metastasis [69], and extra-pelvic recurrence [70]. More importantly, E-cadherin expression is inversely correlated with survival in endometrial cancer [66, 68-72]. Interestingly, the expression of E-cadherin is

reduced in type II compared to type I endometrial carcinoma, suggesting its loss could contribute to the aggressive behavior of type II endometrial cancers [65, 68, 69, 71, 73].

1.5.3 Transcription factor SNAIL in cancer cell invasion and E-cad expression

SNAIL is a zinc-finger transcription factor which is one of the most prominent E-cadherin repressor. SNAIL mutant mice die at gastrulation because of deregulated EMT and sustained E-cadherin expression [74]. SNAIL overexpression occurs in multiple human cancers and is linked to poor prognosis [75], whereas its down-regulation attenuates tumor growth and invasiveness in animal models [76]. In endometrial cancer, elevated SNAIL expression is related to advanced stage, myometrial invasion and lymph node metastasis [68, 77]. Interestingly, non-endometrioid tumors appear to have higher rates of nuclear SNAIL expression than endometrioid tumors [68], which may contribute to their metastatic propensity.

1.6 PTEN

PTEN is a phosphatase that can act on both polypeptide and phosphoinositide substrates. It's mainly located in cytoplasm or membrane, although its nuclear localization has also been described with important functions. PTEN is a classical tumor suppressor which regulates the phosphatidylinositol 3 kinase (PI3K)/AKT cascade. PI3K is a lipid kinase activated by receptor tyrosine kinases, G protein-coupled receptors and RAS activation. It can convert the lipid second messenger phosphatidylinositol 4,5-biphosphate (PIP2) to phosphatidylinositol 3,4,5-trisphosphate (PIP3); PIP3 then recruits phosphatidylinositol-dependent kinase 1 (PDK1) and AKT to the plasma membrane, where AKT is phosphorylated by PDK1 and mTORC2. PTEN

antagonizes the action of PI3K-AKT-mTOR pathway by dephosphorylating PIP3 to PIP2 and affects cell cycle progression, apoptosis, angiogenesis, migration and etc [78].

1.6.1 PTEN in cancer

PTEN was first identified to be frequently disrupted in multiple sporadic tumour types and targeted by germline mutations in patients with cancer predisposition syndromes such as Cowden disease. Afterwards, knockout of PTEN in mouse models demonstrates the essential tumor suppressive role of PTEN in multiple cancer types [79]. Importantly, PTEN function is associated with the level of expression or activity of the PTEN protein rather than a discrete set of step-wise changes in the PTEN gene copy number [80, 81]. The subtle down-regulation of PTEN levels or partial loss of PTEN function can result in tremendous functional consequences such as increased cancer susceptibility and tumour progression [81]. The expression and function of PTEN can be regulated by several mechanisms including genetic mutation, transcriptional or post-transcriptional regulation, post-translational regulation, and protein-protein interactions [78].

1.6.2 Expression and function of PTEN in endometrium

PTEN mutation occurs in up to 80% of type I endometrioid cancer but only up to 11% of type II cancer [82]. PTEN heterozygous mice develop endometrioid tumors and PTEN mutations have also been detected in around 55% of endometrial hyperplasia cases, indicating its early role in the pathogenesis of endometrioid cancer [7, 83]. Although PTEN genetic mutation is relatively rare in type II endometrial cancer, its expression at protein level might be down-regulated by TGF β 1 which is abundantly expressed in all grades of endometrial cancer [84]. This hypothesis is supported by the study where the protein expression level of PTEN is down-regulated by

TGF β 1 treatment in a type II endometrial cancer cell line KLE [85]. However, to date, the function of PTEN and its regulation by TGF β 1 in type II endometrial cancer have not been fully characterized.

Chapter 2: Rationale and objectives

2.1 Rationale

Endometrial cancer is the most common, and second most lethal, gynecological malignancy and the fourth most common female cancer in North America [8]. Type II endometrial cancers are predominantly non-endometrioid (serous and clear cell) carcinomas associated with advanced stage and poor survival [7, 83, 86]. In particular, despite accounting for only ~10% of all endometrial cancers, serous endometrial carcinomas are responsible for ~40% of deaths due to their high grade, deep myometrial invasion and propensity for extra-uterine spread [7, 83, 87]. Moreover, the number of endometrial cancer related deaths has been growing [8], which can be attributed to the increased incidence of advanced-stage tumor and high risk histologies [88]. From a clinical perspective, the extent of myometrial invasion and metastasis is the most important prognostic factor in endometrial cancer [89]. However, the key molecular events which trigger endometrial cancer invasion and dissemination still remain delineated [90].

Activin B is a disulfide-linked homodimer of inhibin β B subunits which belong to the transforming growth factor-beta (TGF β) superfamily [91]. While TGF β s have been well characterized for their roles in development and EMT during cancer, particularly regarding cell migration and invasion, activin signaling is less well documented [31]. In endometrial cancer, early studies demonstrated inhibin β subunit expression, activin secretion and activin receptor expression in neoplastic endometrial tissues and/or endometrial cancer cell lines [33, 34, 92]. Importantly, elevated inhibin β B is associated with reduced cause specific survival in non-endometrioid tumors (~70% serous) [36], indicating activin B may be linked to the poor survival

of the most lethal subtype of endometrial cancer. However, few studies have examined the effects and molecular mechanisms of activin B on endometrial cancer cells.

TGF β s are similar to activins in that they not only show structural similarity but both operate through canonical SMAD2/3-SMAD4 and non-canonical signalings, such as AKT/PI3K, MAPK/ERK and Wnt/ β -catenin. Despite their overlapping SMAD-dependent and independent pathways, activins and TGF β s signal through common, as well as different, downstream transcriptional targets, resulting in different functional consequences [31]. For example, TGF β inhibits trophoblast invasion while activin A can promote trophoblast invasion via MMPs [5]. Importantly, whether TGF β and activin share similar functions and molecular mechanisms in endometrial cancer development remains unknown. Histopathological and functional studies suggest that TGF β signalings are closely associated with the neoplastic transformation of human endometrium and the initiation of endometrial cancer invasion. There is a statistically significant gradual increase in the immune-staining of all three TGF β s progressing from the normal proliferative endometrium to simple hyperplasia and on to complex hyperplasia [37]. Consistently, TGF β 1 plasma level is greater in Stage-Ib and Stage-Ic patients than in Stage-Ia patients [38]. TGFBR2 protein level estimated by ELISA is higher in endometrial cancer compared to normal endometrial tissues [39]. Significantly elevated protein level of TGFBR2 is also noted in endometrial cancer with myometrial invasion compared to those non-infiltrating tumors [39, 40]. Moreover, the expression of TGF β signaling effectors SMADs has been deregulated, indicating the switch of TGF β 1 from a tumor suppressor to a tumor promoter in endometrial cancer [93]. Indeed, gene expression profiling of high-risk recurrence endometrial cancer identifies TGF β 1 as the core factor in the initiation of tumor invasion. Consistently, in

vitro experiments show that TGF β 1 can promote the invasiveness of KLE, HEC-1A and RL95-2 endometrial cancer cells [38, 41]. Although much has been documented about the expression and roles of TGF β 1 signaling in endometrial cancer, the mechanism of TGF β 1 that governs the migration, the initial stage of metastasis of advanced endometrial cancer, is not well understood.

The levels of integrin expression and integrin affinity for ECM substrates have always been dramatically altered as the tumor progresses. The notable differences in cell surface expression and distribution of integrins in malignant tumors compared with pre-neoplastic tumors of the same type have been documented in numerous cancers [43]. Specifically, integrin α v β 3 is expressed in endometrioid and serous endometrial cancers [50, 51]. However, its clinical and/or therapeutic relevance in these tumors has yet to be defined.

Apart from integrin-mediated cell-matrix contact, the metastatic capacity of tumors is also governed by cadherin mediated cell-cell adhesion [94]. In particular, cancer cell metastasis is often associated with epithelial-mesenchymal transition which is characterized by the down-regulation of E-cadherin [95]. Increasing evidences suggest that endometrial cancers display a number of features associated with the epithelial-mesenchymal transition process [96]. For examples, reduced E-cadherin expression is associated with advanced stage [65, 97], poor differentiation [65, 67], deep myometrial invasion [67, 68, 97], lymph node metastasis [69], and extra-pelvic recurrence [70]. More importantly, E-cadherin expression is inversely correlated with survival in endometrial cancer [68-72, 97]. Interestingly, the expression of E-cadherin is reduced in type II compared to type I endometrial carcinoma, suggesting its loss could contribute to the aggressive behavior of type II endometrial cancers [65, 68, 69, 71, 73]. SNAIL is a well-

known transcriptional repressor of E-cadherin that is overexpressed in multiple human cancers [75]. In endometrial cancer, elevated SNAIL expression is related to advanced stage, myometrial invasion and lymph node metastasis [68, 77]. Interestingly, non-endometrioid tumors appear to have higher rates of nuclear SNAIL expression than endometrioid tumors [68], which may contribute to their metastatic propensity. However, the exact roles and upstream regulators of E-cadherin and SNAIL in endometrial cancer are not well-defined;

PTEN (phosphatase and tensin homologue) is a well-known tumor suppressor mutated in multiple sporadic tumor types. Loss of PTEN function would cause hyper-activation of PI3K-AKT pathway that regulates cell growth, apoptosis, invasion and metastasis [78]. Moreover, even subtle repression of PTEN expression levels could have tremendous consequences on tumor progression [81]. Although PTEN mutation is relatively rare in type II endometrial cancer [83], the rate of PTEN protein loss in type II endometrial cancer is much higher than alterations in the PTEN gene [98, 99]. These findings indicate that loss of PTEN expression can be achieved by transcriptional or post-translational modulations. This is supported by a study where TGF β 1 decreases PTEN protein expression by increasing its degradation in a type II endometrial cancer cell line KLE [100]. However, to date, the function of PTEN and its regulation by TGF β 1 in type II endometrial cancer have not been fully characterized [78, 101].

2.2 Overall hypothesis

The overall hypothesis of this study is that activin B and TGF β 1 promote type II endometrial cancer cells adhesion or migration by regulating related key tumorigenic factors.

2.3 Aim of the study

The general aim of this study is to investigate the role of activin B and TGF β 1 in type II endometrial cancer migration as well as their underlying molecular mechanisms.

2.4 Study models

The KLE, HEC-1B and HEC-50 cell lines are used as study models, which have been used widely as models representing type II endometrial cancer [15, 16, 21-23]. KLE cells were derived from a poorly differentiated metastatic endometrial carcinoma in a 64-year old patient [16, 20]. HEC-1B cells were derived from tumor tissues of a 71-year-old female with Grade 2 endometrial carcinoma [18]. HEC-50 was obtained from the ascites of a patient with recurrent Grade 3 cancer [19].

2.5 The specific objectives of this study

Objective 1: To investigate the role of activin B in type II endometrial cancer adhesion, migration and invasion. (Presented in Chapter 3)

- (1) To investigate the effects of activin B on endometrial cancer cell adhesion, migration and invasion.
- (2) To determine the effects of activin B on integrin α v β 3 expression.
- (3) To investigate the effects of activin B on SMAD signaling pathway.
- (4) To investigate the SMAD2/3-SMAD4 signaling mediated up-regulation of integrin β 3.
- (5) To examine the role of integrin β 3 in activin B-regulated cell adhesion, migration and invasion.

Objective 2: To investigate the involvement of E-cadherin in activin B induced type II endometrial cancer migration. (Presented in Chapter 4)

- (1) To determine the effects of activin B on E-cadherin expression.
- (2) To investigate the effects of activin B on SMAD signaling pathway.
- (3) To investigate the SMAD2/3-SMAD4 signaling in activin B mediated down-regulation of E-cadherin.
- (4) To investigate the effects of activin B on SMAD independent signaling pathways.
- (5) To examine the role of MEK-ERK1/2 signaling in activin B mediated down-regulation of E-cadherin.
- (6) To investigate the effects of activin B and MEK-ERK1/2 signaling on SNAIL expression.
- (7) To identify the role of E-cadherin in activin B induced cell migration.

Objective 3: To investigate the role of integrin $\alpha v \beta 3$ in TGF β 1 mediated-endometrial cancer cell adhesion and migration. (Presented in Chapter 5)

- (1) To investigate the effects of TGF β 1 on endometrial cancer cell adhesion and migration
- (2) To determine the effects of TGF β 1 on integrin $\alpha v \beta 3$ expression.
- (3) To investigate the effects of TGF β 1 on SMAD signaling pathway.
- (4) To investigate the SMAD2/3-SMAD4 signaling in TGF β 1 mediated up-regulation of integrin αv and $\beta 3$.
- (5) To examine the role of MEK-ERK1/2 signaling in TGF β 1 mediated up-regulation of integrin αv and $\beta 3$.
- (6) To identify the role of integrin $\beta 3$ or αv in TGF β 1-regulated cell adhesion and migration.

Objective 4: To investigate the role of PTEN in TGF β 1 mediated-endometrial cancer cell migration. (Presented in Chapter 6)

- (1) To determine the effects of TGF β 1 on PTEN expression.
- (2) To identify the involvement of PTEN in TGF β 1-regulated cell migration.
- (3) To investigate the effect of PTEN overexpression on TGF β 1 induced AKT phosphorylation.
- (4) To investigate the effects of TGF β 1 on SMAD signaling pathway.
- (5) To investigate the SMAD2/3-SMAD4 signaling in TGF β 1 mediated down-regulation of PTEN
- (6) To examine the role of MEK-ERK1/2 signaling in TGF β 1 mediated down-regulation of PTEN

Chapter 3: Activin B induces human endometrial cancer cell adhesion, migration and invasion by up-regulating integrin $\beta 3$ via SMAD2/3 signaling

3.1 Introduction

Endometrial cancer is the most common, and second most lethal, gynecological malignancy and the fourth most common female cancer in North America [8]. Traditionally, endometrial cancers have been broadly classified into two clinicopathological types [102]. Accounting for ~70% of endometrial cancers, type I tumors are primarily comprised of low-grade endometrioid carcinomas associated with unopposed estrogen and favorable prognosis. In contrast, type II endometrial cancers are predominantly non-endometrioid (serous and clear cell) carcinomas associated with advanced stage and poor survival [7, 86]. In particular, despite accounting for only ~10% of all endometrial cancers, serous endometrial carcinomas account for ~40% of deaths due to their high grade, deep myometrial invasion and propensity for extrauterine spread [7, 87]. In The Cancer Genome Atlas's recent genomic characterization of endometrial carcinomas, serous tumors and ~25% of high-grade endometrioid tumors were grouped in a novel genomic class (copy-number high, serous-like) characterized by extensive copy number alterations, frequent TP53 mutations, and poor outcome [11].

Activins are disulfide-linked homodimers of inhibin β subunits which belong to the transforming growth factor-beta (TGF- β) superfamily [91]. The primary isoforms of activins are activin A ($\beta A\beta A$), activin AB ($\beta A\beta B$) and activin B ($\beta B\beta B$). Activins are expressed in many reproductive tissues, including the endometrium, where they regulate numerous biological functions in an autocrine/paracrine manner [103]. In humans, transcripts encoding inhibin βA and βB subunits as well as activin receptors have been detected in primary cultures of normal endometrial

epithelial and stromal cells [33]. At the protein level, secreted activin A has been detected in conditioned medium from normal endometrial epithelial and stromal cells [33], and immunohistochemical analyses have confirmed endometrial expression of inhibin β A and β B subunits throughout the human menstrual cycle [104].

Additionally, increasing evidence suggests that activins and their receptors may participate, either positively or negatively, in the development or progression of a variety of endocrine-related cancers [26]. In endometrial cancer, early studies demonstrated inhibin β subunit expression, activin secretion and activin receptor expression in neoplastic endometrial tissues and/or endometrial cancer cell lines [33, 34, 92]. Histopathological studies have since examined the expression of inhibin β A and β B subunits in sizeable cohorts of endometrial carcinomas of either endometrioid [105, 106] or non-endometrioid [36] histology. In endometrioid tumors, positive immunostaining for inhibin β A or β B was correlated with higher grade, though neither subunit was associated with overall, progression free or cause specific survival [105, 106]. In contrast, non-endometrioid tumors (~70% serous) more frequently displayed positive immunostaining for inhibin β A or β B [106]; however, only elevated inhibin β B was associated with reduced cause specific survival [36]. Interestingly, double immunofluorescence staining of endometrioid tumors showed marked co-localization of inhibin α and β A subunits (suggesting production of inhibin A), whereas there was minimal co-localization of inhibin α and β B, suggesting production of activin B [107].

Though histopathological studies suggest activin B may be linked to poor survival in the most lethal subtype of endometrial cancer, few studies have examined the effects of activins on

endometrial cancer cells, and all have examined only the effects of activin A. Early studies demonstrated both pro- and anti-proliferative effects of activin A on HEC-50 and ISH endometrial cancer cells, respectively [92]. However, subsequent studies with HEC-1, HHUA and Ishikawa endometrial cancer cells failed to show any effects of activin A on cell proliferation [34, 108]. In the present study, we examined the effects of activin B on endometrial cancer cell proliferation, migration, invasion and adhesion. We show that activin B does not affect the viability of HEC-1B and KLE type II endometrial cancer cells. However, our results reveal an important role for activin B in promoting HEC-1B and KLE cell migration, invasion and adhesion to vitronectin. In addition, we show that the effects of activin B on cell migration, invasion and adhesion to vitronectin are mediated by the SMAD2/3-SMAD4-dependent up-regulation of integrin $\beta 3$. Our findings suggest that activin B signaling could promote the invasion and/or metastasis of type II endometrial cancers, thereby contributing to poor patient survival.

3.2 Results

Activin B increases endometrial cancer cell migration, invasion and adhesion

In a previous study of 41 non-endometrioid tumors (29 serous, 7 clear cell and 5 undifferentiated), positive immunostaining for inhibin βB was observed in approximately half of the cases and was associated with reduced cause specific survival ($P = 0.026$) and trends towards reduced progression free ($P = 0.111$) and overall ($P = 0.166$) survival [36]. Similarly, Kaplan-Meier analysis of endometrial cancers with serous histology from TCGA ([11]; $n=53$) shows that samples with inhibin βB mRNA levels greater than the median are significantly associated with reduced disease free survival (Log-rank $P = 0.021$, Figure 3.1A) and a trend towards reduced

overall survival (Log-rank $P = 0.094$, Figure 3.1B). Together, these studies suggest that activin B ($\beta\beta$ dimer) signaling could contribute to poor survival in type II serous endometrial cancer.

Next, we examined the biological functions of activin B in two type II endometrial cancer cell lines (HEC-1B and KLE). Transwell migration and Matrigel invasion assay results showed that both HEC-1B and KLE cells exhibited basal levels of cell motility and invasiveness (Figures 3.2A and 3.2B). Importantly, treatment with 50 ng/mL activin B significantly increased cell migration and invasion in both cell lines (Figures 3.2A and 3.2B). In addition, we examined the effects of activin B on cell adhesion to different extracellular matrix proteins. As shown in Figure 3.2C, HEC-1B cell adhesiveness was increased in vitronectin-, fibronectin-, Matrigel- or collagen IV-coated tissue culture plates compared to uncoated plates. Interestingly, treatment with 50 ng/mL activin B significantly enhanced the adhesion of HEC-1B cells to vitronectin, but did not affect adhesion to the other extracellular matrix proteins or uncoated plates (Figure 3.2C). Similarly, activin B treatment increased the adhesion of KLE cells to vitronectin (Figure 3.2C). MTT assay was used to investigate if the effects of activin B on HEC-1B and KLE cell migration, invasion and adhesion could result from changes in cell viability/proliferation. As shown in Figure 3.2D, treatment with 50 ng/mL activin B every 24 h for up to 72 h did not affect HEC-1B or KLE cell viability.

We also pre-treated HEC-1B and KLE cells with the inhibitor SB431542 to determine whether activin/TGF β type I receptors were required for the biological functions of activin B. As shown in Figure 3.3, pre-treatment with SB431542 completely abolished the effects of activin B on cell migration, invasion and adhesion to vitronectin.

Activin B up-regulates integrin $\beta 3$ but not integrin αv

Given that activin B specifically enhanced endometrial cancer cell adhesion to vitronectin, we next examined its effects on the levels of integrin $\alpha v\beta 3$, well known to be a major receptor for vitronectin [109]. As shown in Figure 3.4A, treatment with activin B for different periods of time did not affect the mRNA levels of integrin αv in HEC-1B or KLE cells. However, activin B treatment for 3 h significantly up-regulated integrin $\beta 3$ mRNA levels and this effect was still observed after 48 h of treatment (Figure 3.4A). Western blot analysis was used to confirm the similar stimulatory effects of activin B on integrin $\beta 3$ protein levels, and to show that they could be abolished by pre-treatment with SB431542 (Figure 3.4B).

SMAD2/3-SMAD4 signaling is required for the up-regulation of integrin $\beta 3$ by activin B

To examine the activation of canonical SMAD2/SMAD3 signaling, HEC-1B and KLE cells were treated with activin B and Western blot was used to measure the levels of phosphorylated SMAD2 and SMAD3 in relation to their total levels. As shown in Figure 3.5A, treatment with activin B for 30 or 60 minutes induced the phosphorylation of SMAD2 and SMAD3 in HEC-1B cells, whereas only SMAD2 phosphorylation was increased in KLE cells. Moreover, activin B-induced phosphorylation of SMAD2 and SMAD3 in HEC-1B cells as well as SMAD3 in KLE cells was blocked by pre-treatment with SB431542 (Figure 3.5B).

Next, we used pre-treatment with siRNA targeting common SMAD4 to investigate the involvement of SMAD signaling in the up-regulation of integrin $\beta 3$ by activin B. As shown in Figure 3.6A, transfection with SMAD4 siRNA significantly reduced endogenous SMAD4 mRNA levels and abolished the up-regulation of integrin $\beta 3$ mRNA by activin B in both HEC-

1B and KLE cells. Similarly, Western blot analysis showed that activin B-induced increases in integrin $\beta 3$ protein levels were abolished by pre-treatment of HEC-1B and KLE cells with SMAD4 siRNA (Figure 3.6B).

SMAD2 and SMAD3 have been shown to mediate TGF β -regulated gene expression both redundantly and differentially depending on the cellular context [110]. Therefore, specific siRNAs targeting SMAD2 or SMAD3 were used to investigate their individual roles in the effects of activin B on integrin $\beta 3$ expression in HEC-1B cells. As shown in Figure 3.7A, transfection with siRNA targeting SMAD2 or SMAD3 significantly reduced their respective mRNA levels and abolished the effects of activin B on integrin $\beta 3$ mRNA. Likewise, Western blot analysis showed that activin B-induced increases in integrin $\beta 3$ protein levels were abolished by pre-treatment of HEC-1B cells with siRNAs targeting SMAD2 or SMAD3 (Figure 3.7B). Interestingly, though activin B only increased SMAD2 phosphorylation in KLE cells, its stimulatory effects on integrin $\beta 3$ mRNA and protein levels were abolished following pre-treatment of KLE cells with siRNA targeting SMAD2 or SMAD3 (Figure 3.7).

Integrin $\beta 3$ mediates activin B-induced cell migration, invasion and adhesion to vitronectin

Pre-treatment with siRNA targeting integrin $\beta 3$ was used to investigate its role in activin B-induced cell migration, invasion and adhesion to vitronectin. As shown in Figure 3.8A, transfection with integrin $\beta 3$ siRNA significantly down-regulated integrin $\beta 3$ mRNA and protein levels in both HEC-1B and KLE cells. Transwell migration and Matrigel invasion assays showed that integrin $\beta 3$ knockdown suppressed the motility and invasiveness of both HEC-1B and KLE cells (Figures 3.8B and 3.8C). In addition, activin B-induced cell migration and invasion were

abolished by pre-treatment with integrin $\beta 3$ siRNA (Figures 3.8B and 3.8C). Similarly, integrin $\beta 3$ knockdown reduced both basal and activin B-induced cell adhesion to vitronectin in HEC-1B and KLE cells (Figure 3.8D).

3.3 Discussion

Previous studies have demonstrated differences between activin A and activin B with respect to expression patterns, receptor/antagonist binding affinities, and biological functions [32, 111-115]. Indeed, inhibin βA (*Inhba*) and βB (*Inhbb*) subunit knockout mice exhibit different phenotypes, and the defects observed in *Inhba* knockout mice are only partially restored by insertion of *Inhbb* [116, 117]. On the other hand, we have recently demonstrated that recombinant activin A, B and AB have similar effects on human ovarian granulosa cell steroidogenesis and placental trophoblast cell invasion [118, 119]. Taken together, these studies suggest that activin A and B could function distinctly or similarly depending on the cellular context. Our results showing that activin B does not affect endometrial cancer cell viability are in agreement with previous studies examining the proliferative effects of activin A [34, 108]. However, we cannot definitively rule out potential effects of activin B on cell proliferation due to the low proliferative rates of HEC-1B and KLE cells and the limited time-course of our studies. Indeed, activin A has been shown to inhibit the proliferation of estrogen-responsive ISH endometrial cancer cells, whereas it enhanced the proliferation of estrogen-insensitive HEC-50 endometrial cancer cells [92]. Interestingly, treatment with estradiol abolished the suppressive effects of activin A on ISH cell proliferation, whereas it had no impact on the effects of activin A in HEC50 cells [92]. These results suggest that the effects of activins could be modified by other hormones or paracrine factors, perhaps differentially depending on the activin isoform or type I

vs. type II carcinomas. In renal cancer cells, treatment with activin B had no effect on proliferation *in vitro*, however inhibin β B subunit knockdown cells formed smaller tumors in xenograft studies [120]. Conversely, activins could differentially modulate the effects of other hormones or paracrine factors. For example, activin A has been shown to reduce the growth inhibitory effects of TGF β on endometrial cancer cell proliferation [108]. Future studies will be required to fully characterize the specific roles and molecular determinants of activin A and B in endometrial cancer cell proliferation.

Commonly confined to the uterus, type I endometrial cancers can often be treated by hysterectomy and have good prognosis. In contrast, the most prevalent type II endometrial cancer, serous endometrial carcinoma, has a relapse rate as high as 50% [121] and accounts for 40% of all endometrial cancer related deaths [87]. This lethality is largely due to its propensity for deep invasion and metastatic spread, thus understanding the molecular mechanisms mediating these processes could lead to new therapeutic approaches for type II endometrial cancers. We now demonstrate, for the first time, that treatment with activin B increases type II endometrial cancer cell migration, invasion and adhesion to vitronectin. To date, only a few studies have examined the effects of activin B on cancer cell motility or adhesion. In renal cancer cells, activin B has been shown to increase cell adhesion and invasion [120, 122], however the molecular mechanisms underlying these effects remain unclear. Together with previous histopathological studies, our results suggest that antagonizing activin B signaling could be a novel approach to the treatment of type II endometrial cancer. However, only a few therapeutic agents targeting the activin system have entered clinical trials (www.clinicaltrials.gov), and none of them are entirely specific for activin signaling [123]. LY-2157299 (Galunisertib) is a small-

molecule inhibitor of activin/TGF β type I receptors (related to SB431542) which is currently under phase 1/2 investigation in hepatocellular, pancreatic and glial cancers. Sotatercept (ACE-011) is an activin receptor type IIA (ACVR2A) Fc fusion protein which is currently under phase 1/2 investigation for the treatment of cancer-associated anemia. Interestingly, an ACVR2B Fc fusion protein (STM 434) has entered phase 1 studies in combination with liposomal doxorubicin in patients with ovarian cancer or other advanced tumors, including endometrial cancer (NCT02262455).

Our results show that treatment of endometrial cancer cells with activin B up-regulates integrin $\beta 3$ mRNA and protein levels without altering the expression of integrin αv . Integrin $\beta 3$ forms only two α - β heterodimers, $\alpha v\beta 3$ and $\alpha IIb\beta 3$, both of which are receptors for vitronectin [124]. However, integrin αIIb mRNA levels are nearly undetectable in HEC-1B and KLE cells, whereas the mRNA levels of integrin αv are at least 10 times higher than those of integrin $\beta 3$ (data not shown). Thus, our results showing that knockdown of integrin $\beta 3$ reduced both basal and activin B-induced cell migration, invasion and adhesion to vitronectin likely indicate that these effects are mediated by integrin $\alpha v\beta 3$. Integrin $\alpha v\beta 3$ is expressed in many types of cancer where it plays important roles in promoting angiogenesis and cancer cell adhesion, migration and invasion [125-131]. Though studies have shown that integrin $\alpha v\beta 3$ is expressed in endometrioid and serous endometrial cancers [50, 51], its clinical and/or therapeutic relevance in these tumors has yet to be defined. Molecular therapies targeting $\alpha v\beta 3$ have achieved positive results such as disease stabilization in advanced solid tumors [132, 133], as well as extended survival in high grade glioma [134, 135]. Interestingly, primary serous endometrial cancer cell migration and adhesion to vitronectin were reduced following treatment with inhibitory anti-integrin αv

antibody [50]. However, endometrial cancers have also been shown to express integrin $\alpha\text{v}\beta 5$ and $\alpha\text{v}\beta 6$ [50, 136], though their functional roles are unknown. Regardless, our study demonstrates that integrin $\beta 3$ (likely $\alpha\text{v}\beta 3$) may constitute a novel therapeutic target in type II endometrial cancers by virtue of its ability to promote basal and activin B-induced cell adhesion, migration and invasion.

Apart from integrin-mediated cell-matrix contact, the metastatic capacity of tumors is also governed by cadherin mediated cell-cell adhesion [94]. In particular, cancer cell metastasis is often associated with epithelial-mesenchymal transition which is characterized by the down-regulation of E-cadherin and up-regulation of N-cadherin [95]. Increasing evidence suggests that endometrial cancers display a number of features associated with the epithelial-mesenchymal transition process [96]. Interestingly, we have recently demonstrated that activin B can stimulate human trophoblast cell invasion by up-regulating N-cadherin expression [119]. Whether modulation of E-cadherin or N-cadherin contributes to activin B-induced cell migration and invasion in endometrial cancer remains unknown and warrants further investigation.

Previous studies have shown that the expression of integrin $\beta 3$ in endometrial cancer cells can be regulated by progesterone [137], macrophage migration inhibitory factor [138], and gonadotropin-releasing hormone [139]. Similar to our results for activin B in endometrial cancer cells, TGF $\beta 1$ has been shown to up-regulate integrin $\beta 3$ in glioma cells [140], lung fibroblasts [141], and breast cancer cells [142], whereas it down-regulated integrin $\beta 3$ in lymphoma cells [143]. Interestingly, our results show that while activin B activated both SMAD2 and SMAD3 in HEC-1B cells, only SMAD2 was activated in KLE cells. At present, we do not know why activin

B failed to increase the levels of phosphorylated SMAD3 in KLE cells. However, it has previously been reported that TGF β isoforms can induce SMAD3 phosphorylation in KLE cells [144], thus it does not appear to be a general defect in SMAD3 phosphorylation. Interestingly, depletion of SMAD3 abolished the stimulatory effects of activin B on integrin β 3 expression in KLE cells, suggesting that SMAD3 phosphorylation is not required or endogenous levels of SMAD3 phosphorylation are sufficient for activin B-induced integrin β 3 expression. Regardless, our siRNA results demonstrate that neither SMAD can compensate for the loss of the other, suggesting both SMAD2 and SMAD3 are required for the up-regulation of integrin β 3 by activin B. Interestingly, knockdown of SMAD3 in lung fibroblasts did not alter TGF β 1-induced integrin β 3 production, whereas it attenuated the up-regulation of integrin β 5 [141]. Instead, c-Src and p38 MAPK signaling were required for the up-regulation of integrin β 3 by TGF β 1 [141]. Future studies will be required to clarify how SMAD-dependent and -independent signaling is integrated at the level of the *ITGB3* promoter.

In summary, our results show that activin B stimulates the migration, invasion and adhesion of type II endometrial cancer cells. Moreover, these effects are mediated by the up-regulation of integrin β 3 production in a SMAD2/3-SMAD4-dependent manner. Our study identifies novel molecular mechanisms that may contribute to the invasion and/or metastasis of type II endometrial cancers.

3.4 Materials and methods

Cell culture

HEC1B and KLE type II human endometrial cancer cell lines were purchased from the American Type Culture Collection (Manassas, VA) [23]. HEC-1B cells were cultured in Minimal Essential Medium (Gibco, Life Technologies, Burlington, ON) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories Inc., Logan, UT). KLE cells were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (Gibco, Life Technologies) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories Inc.). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air.

Antibodies and reagents

Rabbit polyclonal anti-human SMAD4 (#9515) antibody was obtained from Cell Signaling Technology. The rabbit monoclonal antibodies used in this study were: human phospho-SMAD2 (Ser465/467; 138D4, Cell Signaling Technology), human phospho-SMAD3 (Ser423/425; C25A9, Cell Signaling Technology), and human SMAD3 (C67H9, Cell Signaling Technology). The mouse monoclonal antibodies used were: human SMAD2 (L16D3, Cell Signaling Technology), human integrin β 3 (#611141, BD Biosciences), human integrin α v (#611012, BD) and sea urchin α -tubulin (B-5-1-2, Santa Cruz). Horseradish peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were obtained from Bio-Rad Laboratories (Hercules, CA). SB431542 was purchased from Sigma-Aldrich (Oakville, ON). Recombinant human activin B was obtained from R&D Systems (Minneapolis, MN).

MTT assay

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma) assay was used to determine cell viability. Cells were seeded one day prior to treatment in 24-well plates (1×10^4 /well) with 500 μ L of medium and then treated with activin B every 24 h for up to 72 h. MTT (final concentration of 0.5 mg/mL) was added at each time point and incubated for 4 h prior to removing the medium and adding DMSO to dissolve the crystals. Absorbances were measured at 490 nm using a microplate spectrophotometer.

Transwell migration and invasion assays

Migration and invasion assays were performed in Boyden chambers with minor modifications [145]. Cell culture inserts (24-well, pore size 8 μ m; BD Biosciences, Mississauga, ON) were seeded with 1×10^5 cells in 250 μ L of medium with 0.1% FBS. Un-coated inserts were used for migration assays whereas inserts pre-coated with growth factor reduced Matrigel (40 μ L, 1 mg/mL; BD Biosciences) were used for invasion assays. Medium with 10% FBS (750 μ L) was added to the lower chamber and served as a chemotactic agent. After incubation for 24 h (migration) or 48 h (invasion), non-migrating/invading cells were wiped from the upper side of the membrane and cells on the lower side were fixed in cold methanol and air dried. Cell nuclei were stained with Hoechst 33258 and counted using a Zeiss Axiophot epifluorescent microscope equipped with a digital camera (QImaging, Surrey, BC). Each individual experiment had triplicate inserts and five microscopic fields (obtained from middle, upper, lower, right and left parts of membrane) were counted per insert using Northern Eclipse 6.0 software.

Adhesion assays

96-well plates were coated overnight at 4°C with vitronectin (1 µg/cm²; R&D Systems), fibronectin (10 µg/cm²; R&D Systems), Matrigel (5 µg/cm²; BD Biosciences) or collagen IV (10 µg/cm²; R&D Systems) and then blocked for 1 h with 0.5% bovine serum albumin. Cells were seeded at a density of 4×10^4 cells/well and incubated at 37°C for 1.5 h. Non-adherent cells were removed by washing with PBS, and adherent cells were fixed with cold methanol and stained with 0.1% crystal violet for 25 minutes at room temperature. After removing the crystal violet solution, the stained cells were washed with water and 10% acetic acid was added to dissolve the crystal violet. Absorbances were measured at 590 nm using a microplate spectrophotometer.

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

Total RNA was extracted using TRIzol reagent (Invitrogen, Life Technologies, Burlington, ON) in accordance with the manufacturer's instructions. Reverse transcription was performed with 2 µg RNA, random primers and M-MLV reverse transcriptase (Promega, Madison, WI). The primers used for SYBR Green RT-qPCR were: integrin β₃, 5'-GAA GGC TGG CAG GCA TTG-3' (forward) and 5'-AAT GAT TGT CAC TAC CAA CAT GAC ACT-3' (reverse); integrin α_v, 5'-TGC CCA GCG CGT CTT C-3' (forward) and 5'-TGG GTG GTG TTT GCT TTG G-3' (reverse); SMAD2, 5'-GCC TTT ACA GCT TCT CTG AAC AA-3' (forward) and 5'-ATG TGG CAA TCC TTT TCG AT-3' (reverse); SMAD3, 5'-CCC CAG CAC ATA ATA ACT TGG-3' (forward) and 5'-AGG AGA TGG AGC ACC AGA AG-3' (reverse); SMAD4, 5'-TGG CCC AGG ATC AGT AGG T-3' (forward) and 5'-CAT CAA CAC CAA TTC CAG CA-3' (reverse) and GAPDH, 5'-GAG TCA ACG GAT TTG GTC GT-3' (forward) and 5'-GAC AAG CTT CCC GTT CTC AG-3' (reverse). RT-qPCR was performed using an Applied Biosystems 7300

Real-Time PCR System equipped with 96-well optical reaction plates. The specificity of each assay was validated by melting curve analysis and agarose gel electrophoresis of the PCR products. Assay performance was validated by assessing amplification efficiencies by means of calibration curves, and ensuring that the plot of log input amount versus ΔC_q has a slope $<|0.1|$. At least three separate experiments were performed and each sample was assayed in triplicate. A mean value of the triplicates was used for the determination of relative mRNA levels by the comparative C_q method with GAPDH as the reference gene and using the formula $2^{-\Delta\Delta C_q}$.

Western blot

Cells were lysed in ice cold lysis buffer (Cell Signaling Technology) with added protease inhibitor cocktail (Sigma-Aldrich). Extracts were centrifuged at 20,000×g for 10 min at 4°C and supernatant protein concentrations were determined using the DC Protein Assay (Bio-Rad Laboratories). Equal amounts of protein (50 µg) were separated by SDS polyacrylamide gel electrophoresis and transferred onto PVDF membranes. After blocking for 1 h with 5% non-fat dry milk in Tris-buffered saline (TBS), the membranes were incubated overnight at 4°C with primary antibodies that were diluted 1000-fold in 5% non-fat milk-TBS. Following primary antibody incubation, the membranes were incubated with the appropriate HRP-conjugated secondary antibody. Immunoreactive bands were detected using enhanced chemiluminescent substrate or SuperSignal West Femto chemiluminescent substrate (Thermo Fisher) and X-ray film. Membranes were stripped with stripping buffer (50 mM Tris-HCl pH 7.6, 10 mM β -mercaptoethanol, and 1% SDS) at 50°C for 30 minutes and reprobed with anti- α -tubulin antibody. Densitometric quantification was performed using Scion Image software (Scion Corp, Frederick, MD) with α -tubulin as the internal control for normalization.

Small interfering RNA (siRNA) transfection

To knock down endogenous integrin $\beta 3$, SMAD2, SMAD3 and SMAD4, forty percent confluent cells were transfected for 48 hours with 20 nM ON-TARGET*plus* SMART pool siRNA targeting human integrin $\beta 3$, SMAD2, SMAD3 and SMAD4 (Dharmacon, Lafayette, CO) using Lipofectamine RNAiMAX (Invitrogen, Life Technologies). ON-TARGET*plus* Non-targeting pool siRNA (Dharmacon) was used as the control.

Statistical analysis

Results are presented as the mean \pm SEM of at least three independent experiments. For experiments involving only two groups, results were analyzed by Two-Sample *t*-test assuming unequal variances using Excel. Multiple group comparisons were analyzed by one-way ANOVA followed by Student-Newman-Keuls test using PRISM software (GraphPad Software). Means were considered significantly different if $P < 0.05$ and are indicated by different letters.

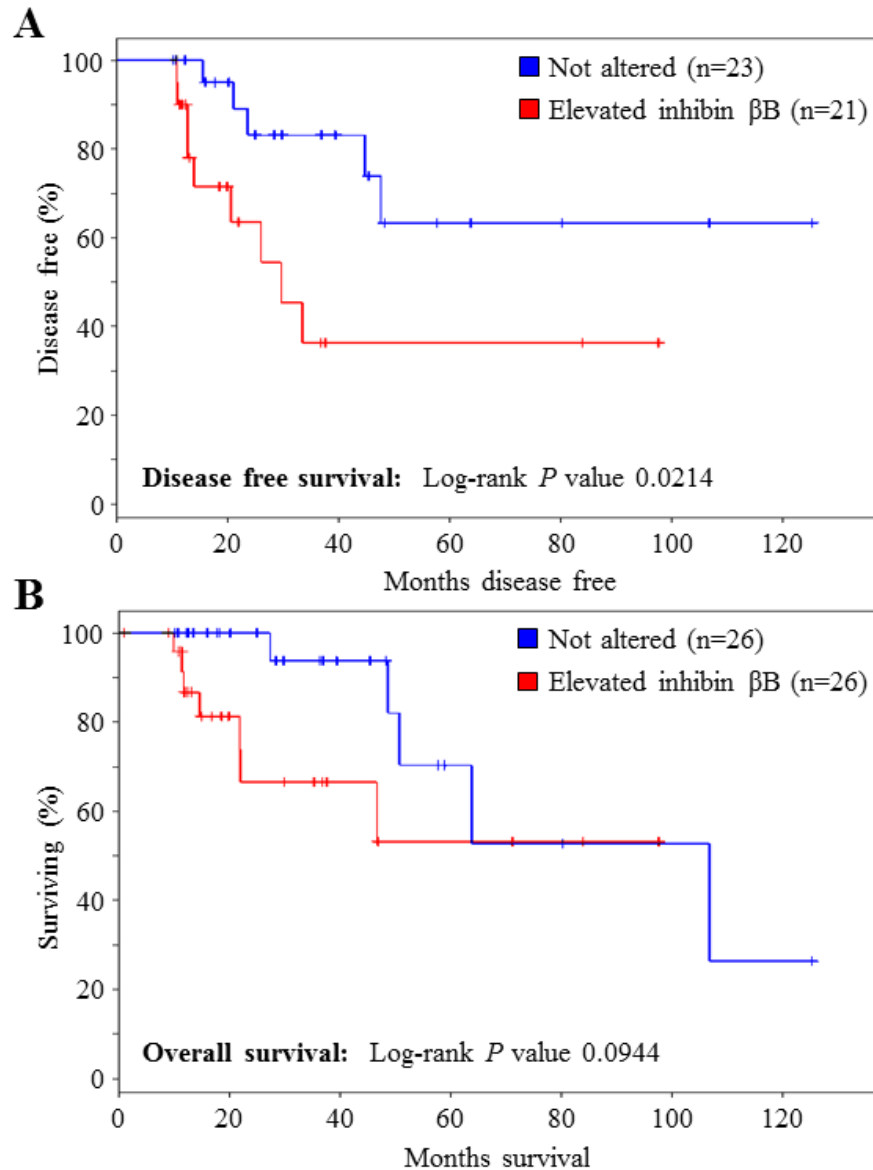


Figure 3. 1 Elevated inhibin β B is associated with reduced disease free survival and a trend towards reduced overall survival in serous endometrial cancers.

The cBioPortal for Cancer Genomics was used to query endometrial carcinomas with serous histology from The Cancer Genome Atlas (n=53) for up-regulation of inhibin β B subunit mRNA above the median. Disease free (A) and overall (B) survival differences between unaltered samples and those with elevated inhibin β B are displayed as Kaplan-Meier survival curves with a P value from a Log-rank test.

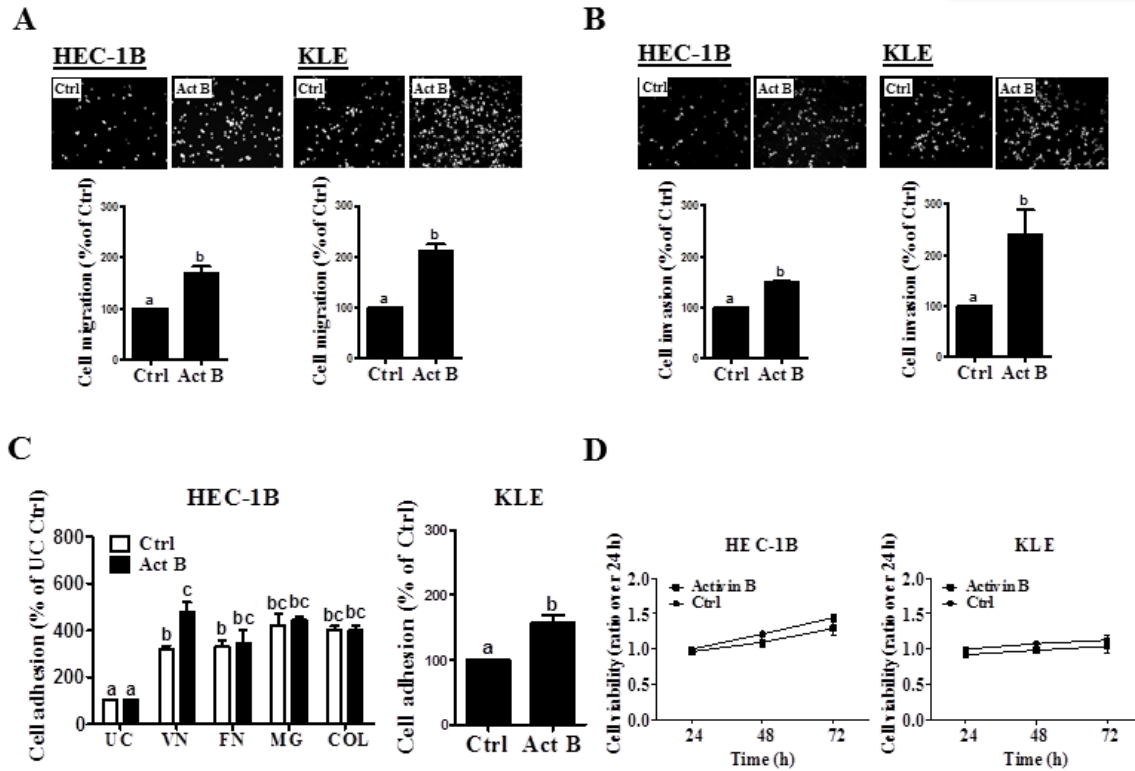


Figure 3. 2 Activin B increases endometrial cancer cell migration, invasion and adhesion.

A and B, HEC-1B and KLE cells were treated without (Ctrl) or with 50 ng/mL activin B (Act B) for 24 h and then seeded in un-coated (A) or Matrigel-coated (B) transwell inserts for migration or invasion assays, respectively. *Upper panels* show representative photomicrographs of migrating/invading cells, while *lower panels* show summarized quantitative results. C, HEC-1B cells were treated with 50 ng/mL activin B for 24 h and then subjected to adhesion assays in un-coated (UC) plates or plates coated with vitronectin (VN), fibronectin (FN), Matrigel (MG) or collagen IV (COL). Additionally, adhesion assays were performed in un-coated or vitronectin-coated plates following treatment of KLE cells with 50 ng/mL activin B for 24 h. D, HEC-1B and KLE cells were treated with 50 ng/mL activin B every 24 h for up to 72 h and cell viability was examined by MTT assay. Results are expressed as the mean \pm SEM of at least three independent experiments. Values without a common letter are significantly different ($P < 0.05$).

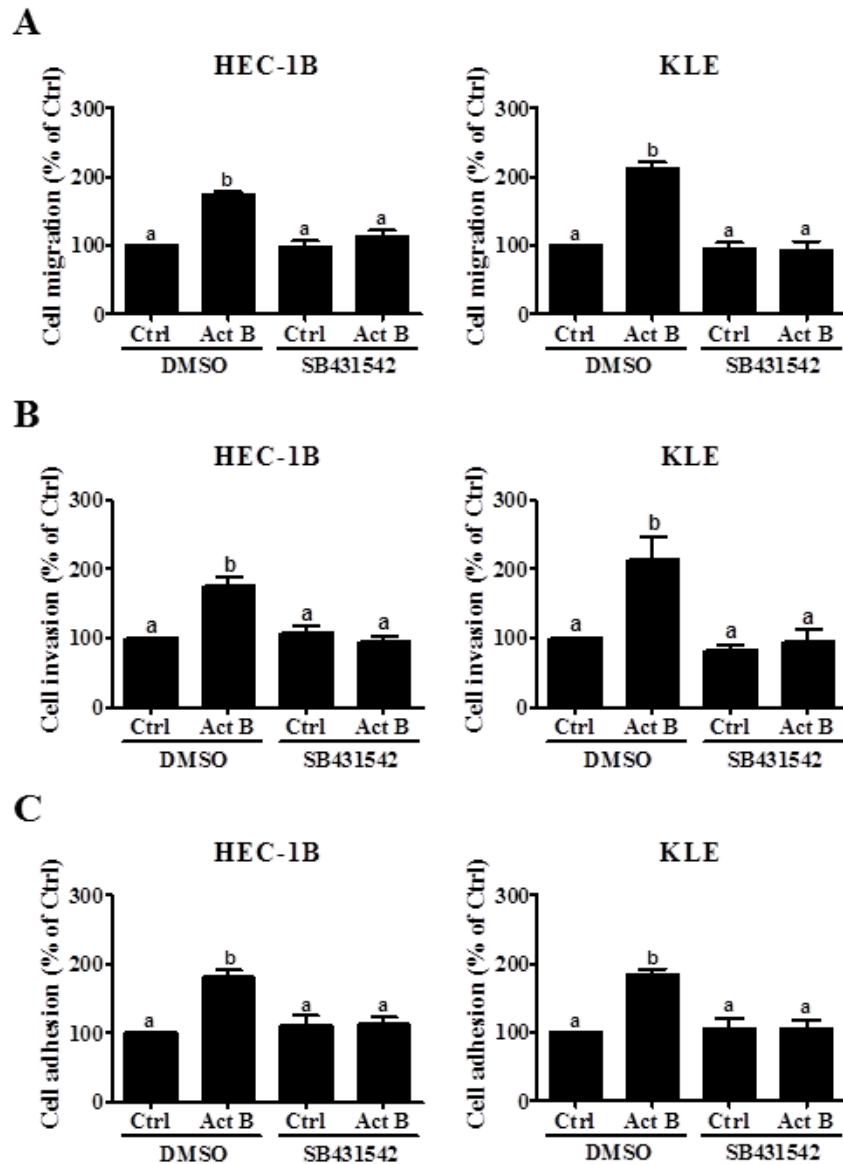
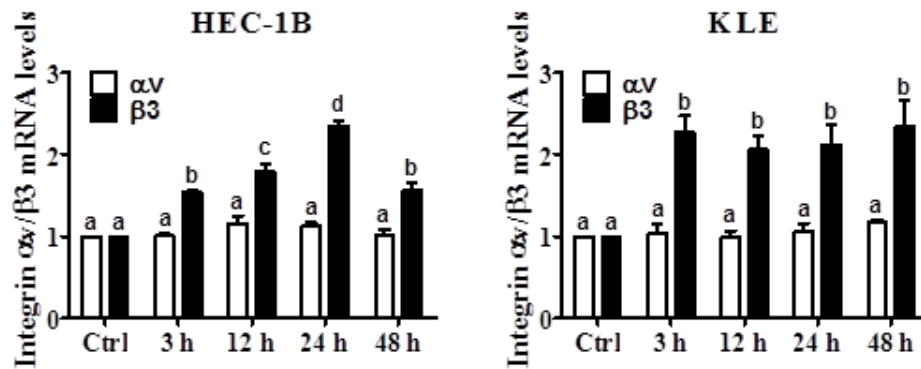


Figure 3. 2 SB431542 abolishes activin B-induced cell migration, invasion and adhesion.

Migration (A), invasion (B) and vitronectin adhesion (C) assays were performed with HEC-1B and KLE cells following pre-treatment with vehicle control (DMSO) or SB431542 (10 μ M) for 1 h prior to treatment without (Ctrl) or with 50 ng/mL activin B (Act B) for a further 24 h. Results are expressed as the mean \pm SEM of at least three independent experiments. Values without a common letter are significantly different ($P < 0.05$).

A



B

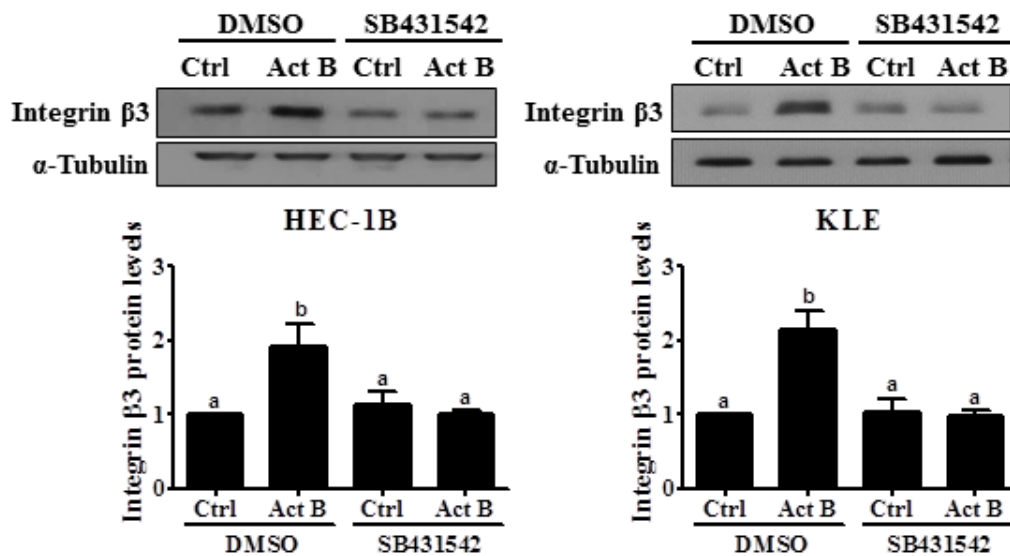


Figure 3. 3 Activin B up-regulates integrin β_3 expression in endometrial cancer cells.

A, HEC-1B and KLE cells were treated for varying times without (Ctrl; time-matched controls displayed as single bar) or with 50 ng/mL activin B (Act B) and integrin α_v and β_3 mRNA levels were measured by RT-qPCR. B, HEC-1B and KLE cells were pre-treated with vehicle control (DMSO) or SB431542 (10 μ M) for 1 h and then treated with 50 ng/mL activin B for 24 h. Protein levels of integrin β_3 were examined by Western blot (quantified data are normalized to α -tubulin) ting. Results are expressed as the mean \pm SEM of at least three independent experiments. Values without a common letter are significantly different ($P < 0.05$).

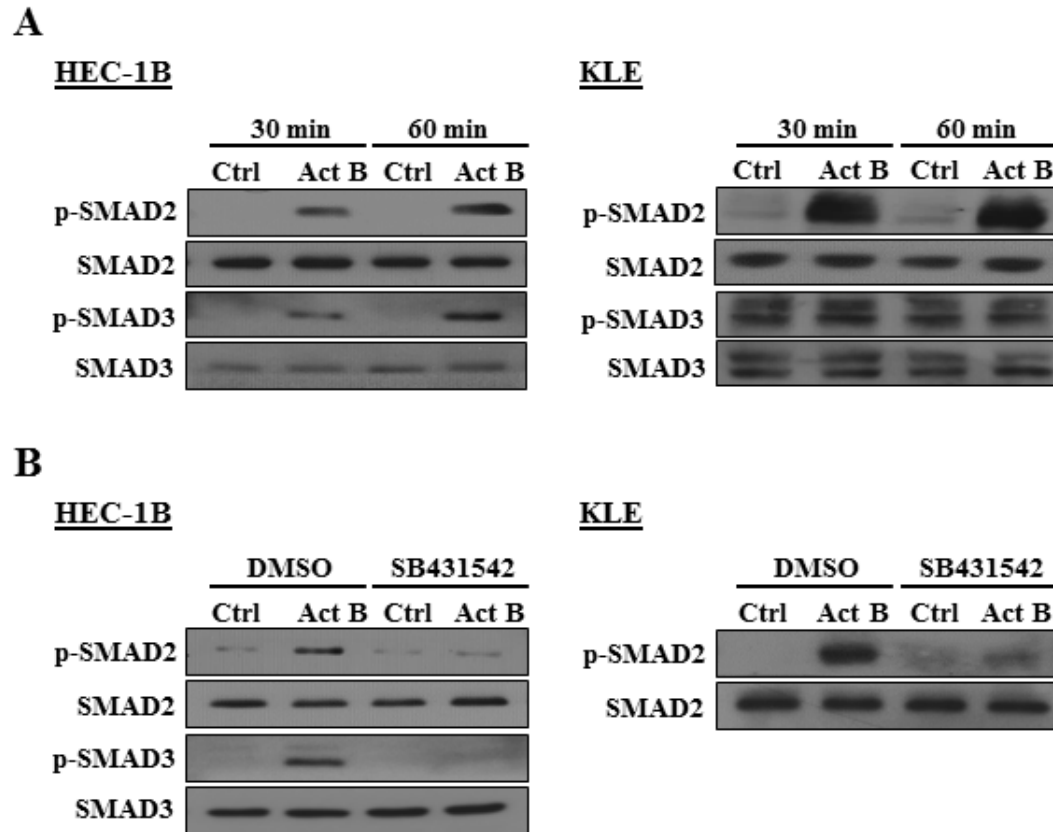


Figure 3. 4 Effects of activin B on SMAD2 and SMAD3 phosphorylation in endometrial cancer cells.

A, HEC-1B and KLE cells were treated without (Ctrl) or with 50 ng/mL activin B (Act B) for 30 or 60 min and Western blot was used to measure the levels of phosphorylated SMAD2 (p-SMAD2) and SMAD3 (p-SMAD3) in relation to their total levels (SMAD2 and SMAD3, respectively). B, HEC-1B and KLE cells were pre-treated with vehicle control (DMSO) or SB431542 (10 μ M) for 1 h and then treated with 50 ng/mL activin B for 60 min. SMAD2 and SMAD3 phosphorylation was examined by Western blot.

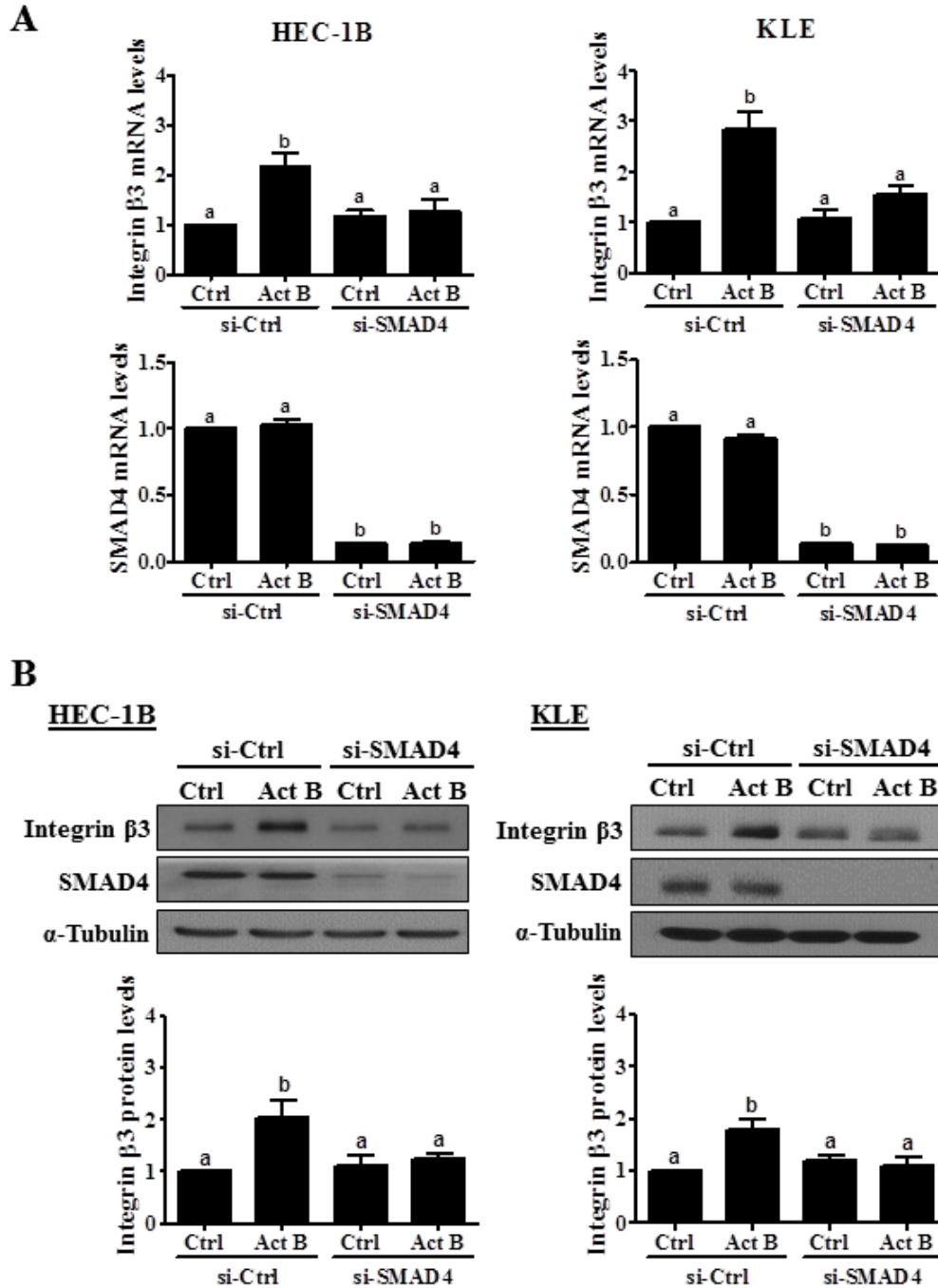


Figure 3. 5 SMAD4 is required for the up-regulation of integrin $\beta 3$ by activin B.

HEC-1B and KLE cells were transfected for 48 h with 20 nM control siRNA (si-Ctrl) or SMAD4 siRNA (si-SMAD4) and then treated without (Ctrl) or with 50 ng/mL activin B (Act B) for 24 h. Integrin $\beta 3$ and SMAD4 mRNA (A) and protein (B) levels were measured by RT-qPCR and

Western blot, respectively. Results are expressed as the mean \pm SEM of at least three independent experiments. Values without a common letter are significantly different ($P < 0.05$).

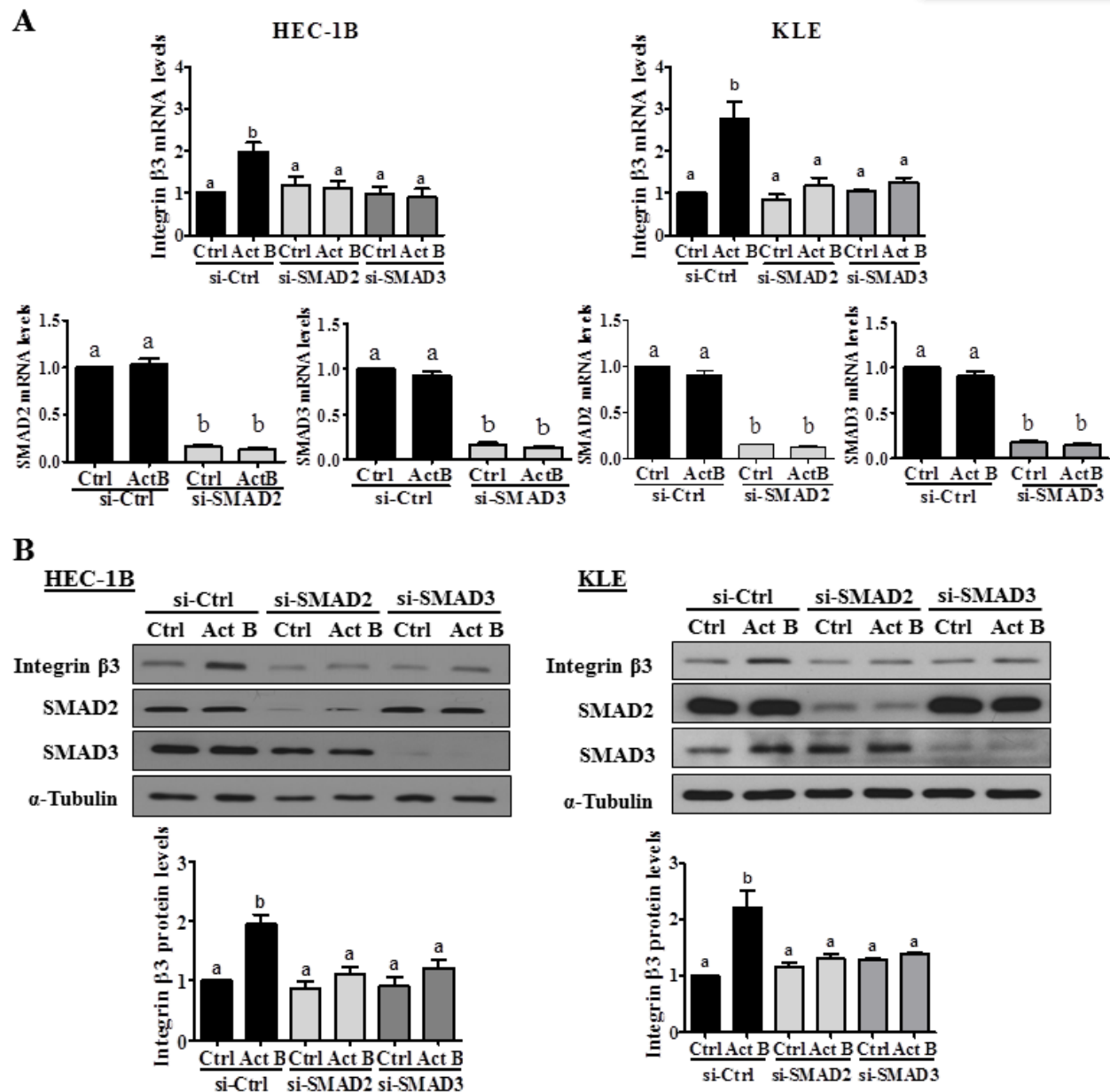


Figure 3. 6 SMAD2 and SMAD3 are required for the up-regulation of integrin $\beta 3$ by activin B.

HEC-1B and KLE cells were transfected for 48 h with 20 nM control siRNA (si-Ctrl), SMAD2 siRNA (si-SMAD2) or SMAD3 siRNA (si-SMAD3) and then treated without (Ctrl) or with 50 ng/mL activin B (Act B) for 24 h. Integrin $\beta 3$, SMAD2 and SMAD3 mRNA (A) and protein (B) levels were measured by RT-qPCR and Western blot, respectively. Results are expressed as the

mean \pm SEM of at least three independent experiments. Values without a common letter are significantly different ($P < 0.05$).

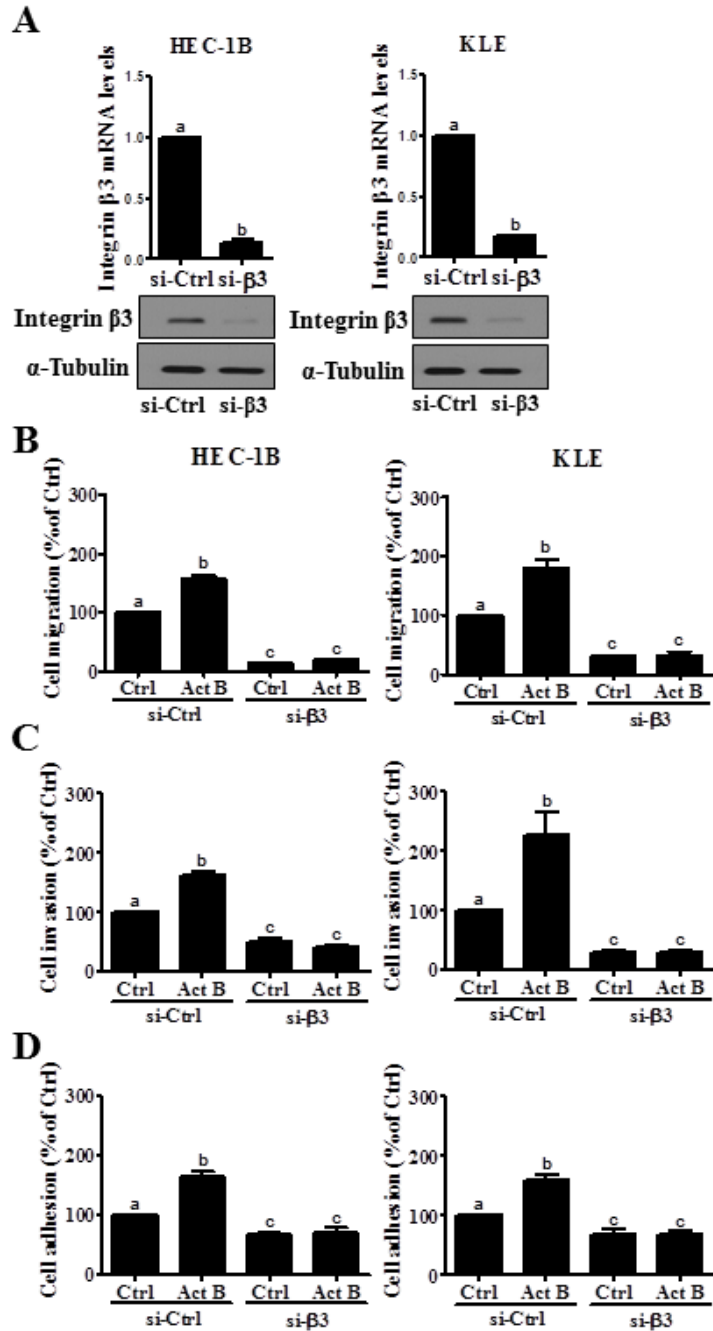


Figure 3. 7 Integrin $\beta 3$ mediates activin B-induced endometrial cancer cell migration, invasion and adhesion to vitronectin.

A, HEC-1B and KLE cells were transfected for 48 h with 20 nM control siRNA (si-Ctrl) or integrin $\beta 3$ siRNA (si- $\beta 3$) and knockdown efficiencies were examined by RT-qPCR and Western blot. Migration (B), invasion (C) and vitronectin adhesion (D) assays were performed with HEC-

1B and KLE cells following transfection with 20 nM control or integrin β 3 siRNA for 48 h prior to treatment without (Ctrl) or with 50 ng/mL activin B (Act B) for a further 24 h. Results are expressed as the mean \pm SEM of at least three independent experiments. Values without a common letter are significantly different ($P < 0.05$).

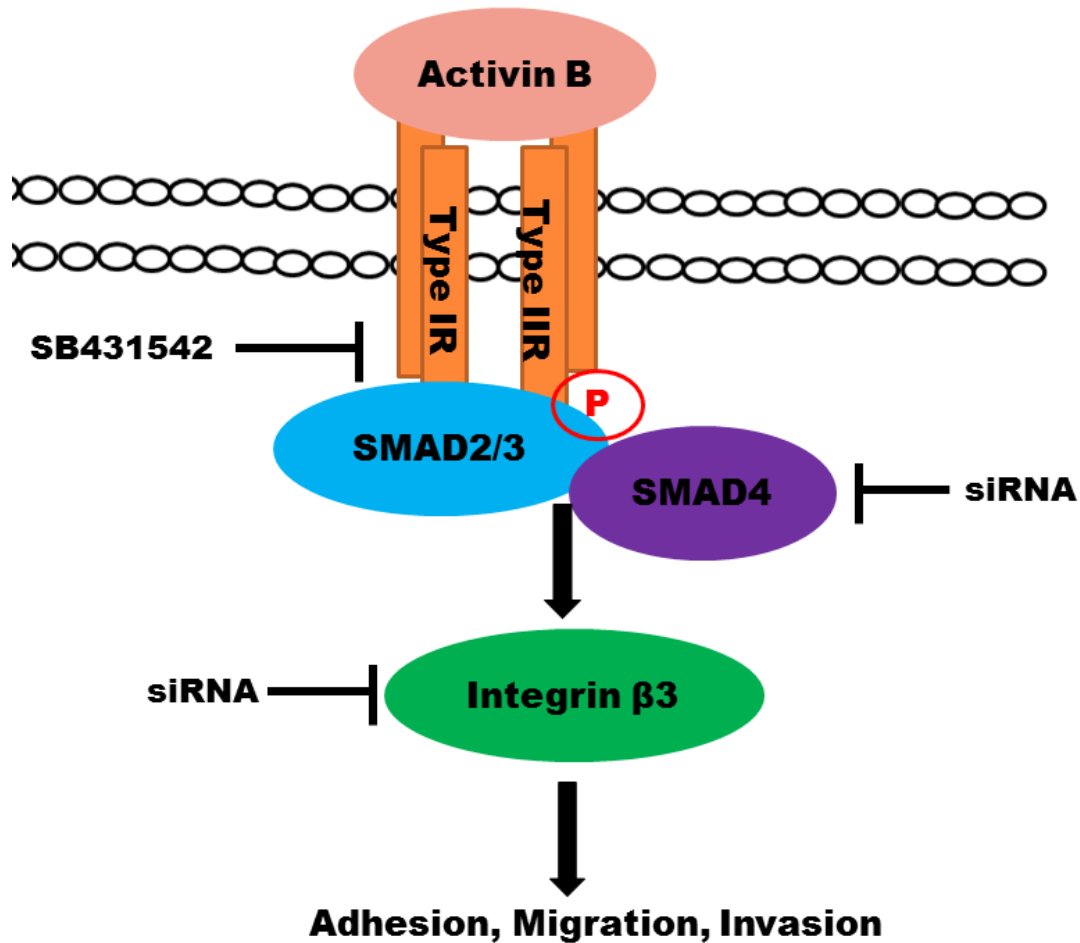


Figure 3. 8 A schematic illustration of Chapter 3 data

Treatment of HEC-1B and KLE cells with Activin B generated comparable increases in cell migration, adhesion and invasion (Figure 3.2), integrin $\beta 3$ expression (Figure 3.4) and SMAD2/3 phosphorylation (Figure 3.5). Co-treatment with the TGF β type I receptor inhibitor SB431542 abolished activin B induced cell adhesion, migration and invasion, up-regulation of integrin $\beta 3$ (Figure 3.3). Knockdown of SMAD2, 3, or 4 (Figure 3.5 and 3.6) reduced the effects of activin B on integrin $\beta 3$. Importantly, activin B induced cell adhesion, migration and invasion were attenuated by siRNA-mediated down-regulation of integrin $\beta 3$ (Figure 3.8).

Chapter 4: Activin B promotes endometrial cancer cell migration by down-regulating E-cadherin via SMAD-independent MEK-ERK1/2-SNAIL signaling

4.1 Introduction

Endometrial cancer is the second most lethal gynecological malignancy in North America. While the mortality rates of many cancers have been effectively reduced, the number of deaths due to endometrial cancer continues to rise [8]. Large, population-based studies of the Surveillance, Epidemiology, and End Results (SEER) database suggest this phenomenon can likely be attributed to the increased incidence of advanced-stage tumors and high risk histologies [88]. Endometrial cancers have traditionally been classified into type I (~75% of cases) or type II tumors as defined by Bokhman [102]. Whereas type I tumors are estrogen-dependent, non-metastatic, and associated with favorable prognosis, type II tumors tend to be estrogen-independent, highly invasive and more lethal. Type I endometrial cancers are generally low-grade endometrioid tumors whereas type II endometrial cancers are mostly non-endometrioid tumors of serous or clear cell histology [83]. Serous endometrial carcinomas account for ~40% of all endometrial cancer deaths and are extremely aggressive, with relapse rates as high as 50% and 5-year overall survival rates as low as 18-27% [83, 87, 121]. Thus, a deeper understanding of the molecular pathways involved in the invasion and metastatic spread of type II endometrial cancers is needed in order to develop new therapeutic approaches with the potential to improve patient outcomes.

Activins belong to the transforming growth factor- β (TGF β) superfamily of cytokines, which includes TGF β s, activins, nodal, inhibins, growth differentiation factors (GDFs), and bone

morphogenetic proteins (BMPs). Activins are homo- or hetero-dimers of inhibin β subunits and the primary isoforms are activin A ($\beta A\beta A$), activin AB ($\beta A\beta B$) and activin B ($\beta B\beta B$). Activins bind to type II transmembrane serine-threonine kinase receptors (ACVR2A or ACVR2B) which, in turn, activate type I receptors (ACVR1B) that phosphorylate and activate canonical SMAD signaling pathways. Activins have also been shown to signal in a SMAD-independent manner via the phosphoinositide 3-kinase (PI3K)/AKT and mitogen-activated protein kinase (MAPK) pathways [31]. Activins are overexpressed and correlated with poor prognosis and survival in a variety of human malignancies [31]. More importantly, inhibin β subunit expression, activin secretion and activin receptor expression have been demonstrated in neoplastic endometrial tissues and/or endometrial cancer cell lines [92, 146, 147]. Histopathological studies of inhibin βA and βB subunit expression in endometrial cancers of endometrioid histology failed to show any association with survival [105, 106]. However in a study of 41 non-endometrioid tumors, of which 70% were serous, positive immunostaining for inhibin βB was associated with reduced cause specific survival and trends towards reduced progression free and overall survival [148]. Similarly, our recent analysis of serous endometrial cancers from TCGA ([11]; n=53) showed that elevated inhibin βB mRNA levels are associated with reduced disease free survival and a trend towards reduced overall survival [149]. These studies support the hypothesis that activin B ($\beta B\beta B$) signaling may be linked to poor survival in type II endometrial cancer. Indeed, we have shown that activin B treatment can promote the adhesion, migration and invasion of type II endometrial cancer cells by up-regulating integrin $\beta 3$ in a SMAD-dependent manner [149].

Cancer cell metastasis is also closely associated with EMT which is characterized by the down-regulation of E-cadherin [95]. E-cadherin is a calcium dependent trans-membrane glycoprotein that plays a key role in the formation of adherens junctions between epithelial cells. E-cadherin

expression is regulated by a group of transcription factors associated with the process of EMT, such as SNAIL, SLUG, TWIST and ZEB1 [57]. Reduced E-cadherin expression or loss of function are correlated with metastasis and adverse clinical outcomes in several types of cancer [58-62], whereas overexpression markedly impairs cancer cell invasiveness [59, 60, 150, 151]. In endometrial cancer, decreased E-cadherin is associated with adverse clinicopathological factors and poor survival. In addition, several studies have reported lower levels of E-cadherin in type II compared to type I endometrial cancers [65, 68, 69, 71, 73], which may explain the more aggressive behavior of type II tumors. However, the exact roles of E-cadherin in type II endometrial cancer and the mechanisms responsible for its down-regulation remain poorly understood. Activin A has been shown to promote the migration of different cell types concomitant with E-cadherin down-regulation [152-154]. In the current study, we investigated the effects of activin B on E-cadherin expression in type II endometrial cancer cells. We demonstrate that activin B suppresses the expression of E-cadherin by up-regulating SNAIL via MEK-ERK1/2 signaling, thereby enhancing KLE and HEC-50 cell migration.

4.2 Results

Activin B down-regulates E-cadherin in human endometrial cancer cells

To investigate the relationship between activin B signaling and E-cadherin in serous endometrial carcinoma (TCGA; n=53; [11]), we performed enrichment analysis comparing E-cadherin levels between unaltered samples and those with elevation of at least one component of the activin B ligand-receptor gene set, including inhibin β B (INHBB), type I receptor ACVR1B, and type II receptors ACVR2A and ACVR2B (Figure 4.1). Serous tumors with INHBB, ACVR1B, ACVR2A or ACVR2B mRNA levels in the upper quartile displayed reduced levels of E-

cadherin protein ($P = 0.039$) and a trend towards reduced levels of E-cadherin mRNA ($P = 0.059$). We also performed similar enrichment analysis in endometrioid cancer subtype. Interestingly, no association was found between the elevated activin B ligand-receptor gene set and the E-cadherin expression (Figure 4.2). These findings suggest that enhanced activin B signaling may contribute to the down-regulation of E-cadherin in type II serous endometrial cancer.

To examine the effect of activin B on E-cadherin expression, we treated KLE and HEC-50 type II human endometrial cancer cell lines with 50 ng/mL activin B for different periods of time (3, 6, 12 or 24 h). As shown in Figure 4.3A, treatment with activin B down-regulated E-cadherin mRNA levels in a time-dependent manner in both KLE and HEC-50 cells, with maximal effects observed 24 h after activin B treatment. Western blot results further confirmed the suppressive effects of activin B on E-cadherin protein levels at 24 h in both cell lines (Figure 4.3B). Furthermore, these reductions in E-cadherin protein were abolished by pre-treatment with the activin/TGF β type I receptor inhibitor SB431542 (Figure 4.3B).

SMAD signaling is not required for activin B-induced down-regulation of E-cadherin

We have previously shown that treatment with activin B phosphorylates/activates SMAD2 and SMAD3 in type II human endometrial cancer cells [149]. To examine the involvement of SMAD signaling in activin B-induced down-regulation of E-cadherin, KLE and HEC-50 cells were transfected with siRNA targeting common SMAD4 prior to treatment with activin B. As shown in Figure 4.4A, despite reducing SMAD4 mRNA levels by more than 80%, pre-treatment with SMAD4 siRNA did not alter the inhibitory effects of activin B on E-cadherin mRNA levels in either cell line. Similarly, Western blot analysis showed that the suppressive effects of activin B

on E-cadherin protein levels were not affected by SMAD4 knockdown (Figure 4.4B). Next, we used specific siRNAs targeting SMAD2 or SMAD3 to further confirm that SMAD signaling is not required for the down-regulation of E-cadherin by activin B in KLE and HEC-50 cells. Whereas transfection with SMAD2 or SMAD3 siRNA significantly reduced their respective protein and mRNA levels by more than 75%, neither siRNA altered the inhibitory effects of activin B on E-cadherin mRNA and protein levels (Figure 4.5).

MEK-ERK1/2 signaling is required for the down-regulation of E-cadherin by activin B

Since the effects of activin B on E-cadherin were not mediated by canonical SMAD signaling, we next investigated whether MEK-ERK1/2, PI3K/AKT or p38 MAPK signaling might be involved. To examine the activation of these pathways, we treated KLE and HEC-50 cells with activin B and used Western blot to measure the levels of phosphorylated ERK1/2, AKT and p38 MAPK in relation to their total levels. Whereas treatment with activin B induced the phosphorylation of ERK1/2 in both cell lines after 10 min, ERK1/2 activation was more prolonged in HEC-50 cells (Figure 4.6A). We then used the MEK inhibitor U0126 to determine whether MEK-ERK1/2 signaling is required for the effects of activin B on E-cadherin in KLE and HEC-50 cells. Pre-treatment with U0126 inhibited both the induction of ERK1/2 phosphorylation (Figure 4.6B) and the down-regulation of E-cadherin (Figure 4.6C) by activin B. In contrast, activin B treatment did not alter the phosphorylation of AKT or p38 MAPK at any of the time-points examined (10, 30 or 60 min; Figure 4.7).

Activin B down-regulates E-cadherin via MEK-ERK1/2-induced up-regulation of SNAIL

Next, we examined the effects of activin B on a set of EMT-related transcription factors previously linked to the down-regulation of E-cadherin (SNAIL, SLUG, TWIST and ZEB1 [57]). Treatment of KLE cells with activin B up-regulated SNAIL mRNA levels at 3 and 6 h, whereas significant increases were observed at 1 and 3 h in HEC-50 cells (Figure 4.8A). Western blot results showed that treatment with activin B for 3 h increased SNAIL protein levels in both KLE and HEC-50 cells (Figure 4.8B). In addition, inhibition of MEK-ERK1/2 signaling by pre-treatment with U0126 abolished the up-regulation of SNAIL protein levels by activin B (Figure 4.8B). In contrast, treatment with activin B did not significantly affect the mRNA levels of SLUG, TWIST or ZEB1 at any of the time-points examined in either cell line (1, 3, 6 or 12 h; Figure 4.9).

SNAIL knockdown was used to investigate its involvement in activin B-induced down-regulation of E-cadherin. As shown in Figure 4.10A, pre-treatment with SNAIL siRNA significantly reduced SNAIL mRNA levels and abolished the inhibitory effects of activin B on E-cadherin mRNA levels in both KLE and HEC-50 cells. Similarly, knockdown of SNAIL suppressed endogenous SNAIL protein levels and completely blocked activin-B-induced down-regulation of E-cadherin protein (Figure 4.10B).

Down-regulation of E-cadherin is required for activin B-induced cell migration

Transwell migration assay results confirmed the pro-migratory effects of activin B on both KLE and HEC-50 cells (Figure 4.11A). Furthermore, these effects of activin B were abolished by inhibition of activin/TGF β type I receptors (SB431542; Figure 4.11B) or MEK-ERK1/2

signaling (U0126; Figure 4.11C). Next, we used transient overexpression of full-length human E-cadherin to further investigate the role of E-cadherin in activin B-induced cell migration. Western blot analysis confirmed the increased production of E-cadherin in KLE and HEC-50 cells transfected with the E-cadherin vector (Figure 4.12A). Importantly, migration assay results showed that forced-expression of E-cadherin attenuated activin B-increased cell migration in both KLE and HEC-50 cells (Figure 4.12B).

4.3 Discussion

Like TGF β 1, activins are thought to play both positive and negative roles in tumor development and progression [155]. Nevertheless, blocking the activin/TGF β pathway has been shown to suppress multiple organ metastases in several types of cancer [156-158]. We have previously shown that activin B enhances the adhesion, migration and invasion of type II endometrial cancer cells in a SMAD-dependent manner [149]. We now describe a novel SMAD-independent pathway contributing to the pro-migratory effects of activin B on type II endometrial cancer cells. These findings could be clinically relevant to type II endometrial cancer, especially the serous subtype, because these cancers are frequently associated with invasion of lymphatic and vascular spaces, lymph node metastases, involvement of other intra-peritoneal structures, and distant recurrences [87, 159, 160]. Characterization of the molecular mechanisms contributing to cell motility and invasiveness could help identify novel therapeutic targets for this most lethal type of endometrial cancer.

Studies suggest that EMT may contribute to endometrial cancer metastasis, in particular the loss of E-cadherin and the activation of transcription factors involved in its repression. Indeed,

reduced E-cadherin expression is associated with advanced stage [65, 97], poor differentiation [65, 67], deep myometrial invasion [67, 68, 97], lymph node metastasis [69], and extra-pelvic recurrence [70] in endometrial cancer. More importantly, E-cadherin expression is inversely correlated with survival in endometrial cancer [68-72, 97]. Interestingly, the expression of E-cadherin is reduced in type II compared to type I endometrial carcinoma, suggesting its loss could contribute to the aggressive behavior of type II endometrial cancers [65, 68, 69, 71, 73]. Though the relationship between TGF β 1 and EMT has been well-studied in a variety of cancers [31], much less is known about the roles of activins, especially with respect to the regulation of E-cadherin. Moreover, most studies have examined only the effects of activin A [152-154], despite increasing evidence suggesting that activin isoforms could function differently depending on the cellular context [161]. To date, only one study in clear cell renal cell carcinoma has described the effects of activin B on cell invasion, however activin B did not alter the expression of E-cadherin or its related transcription factors in these cells [122]. We report for the first time that activin B down-regulates E-cadherin in type II endometrial cancer cells, and that forced-expression of E-cadherin reverses activin B-induced cell migration. These cellular effects are consistent with our TCGA analysis showing reduced E-cadherin levels in serous endometrial cancers with elevation of at least one component of the activin B ligand-receptor gene set. Interestingly, no significant differences in E-cadherin protein ($P = 0.238$) or mRNA ($P = 0.606$) levels were observed when similar enrichment analyses were performed with endometrioid endometrial cancers (TCGA; $n=307$; INHBB, ACVR1B, ACVR2A or ACVR2B mRNA levels in the upper quartile). Moreover, in contrast to serous tumors, where increased inhibin β B immunostaining or mRNA levels are associated with reduced survival [148, 149], neither inhibin β A nor β B expression levels are associated with survival in endometrioid endometrial

carcinomas [105, 106]. These findings suggest the relationship between activin B signaling and E-cadherin may be specific to serous endometrial cancer, and may contribute to its aggressive behavior. Our findings suggest that further investigations of activin B signaling or E-cadherin as prognostic biomarkers or therapeutic targets in type II endometrial carcinoma are warranted.

SNAIL is a well-known transcriptional repressor of E-cadherin that is overexpressed in multiple human cancers [75]. Down-regulation of SNAIL attenuates tumor growth and invasiveness in animal models, and its expression is linked to poor prognosis [76]. In endometrial cancer, elevated SNAIL expression is related to advanced stage, myometrial invasion and lymph node metastasis [68, 77]. Interestingly, non-endometrioid tumors appear to have higher rates of nuclear SNAIL expression than endometrioid tumors [68], which may contribute to their metastatic propensity. Up-stream regulators of SNAIL expression in endometrial cancer are not well-defined; however, our study shows that SNAIL can be up-regulated by activin B-induced MEK-ERK1/2 signaling in type II endometrial cancer cells. Moreover, we show that SNAIL is required for activin-B induced down-regulation of E-cadherin. Our findings are consistent with a previous report showing SNAIL up-regulation via ERK1/2-mediated activation of AP-1 transcription factor [76]. ERK signaling also mediates epidermal growth factor-induced SNAIL up-regulation and subsequent cadherin switching and cell invasion in serous borderline ovarian tumor cells [162]. However, the regulation of SNAIL is cell context-dependent as its expression is also governed by SMADs [163] and PI3K-AKT signaling [76].

Activation of SMAD-independent pathways, including MAPK signaling, is well-described for TGF β [30, 164]. These pathways exert their own independent functions however they can also compliment or antagonize canonical SMAD-dependent signaling [30]. On the other hand, far

fewer studies have described SMAD-independent signaling pathways activated by activins. Given that TGF β s and activins utilize distinct sets of type I and type II receptors, they may each activate a unique complement of SMAD-independent signaling pathways which could result in different functional consequences [31]. Several studies have demonstrated that activins can induce MEK-ERK1/2 signaling, and our finding that MEK inhibition blocks activin A-induced endometrial cancer cell migration is consistent with previous studies in mesothelioma and mesenchymal stem cells [165-167]. However unlike the present study, those previous studies did not rule out SMAD-dependent actions/crosstalk. This is important because multiple MAPKs, including ERK1/2, can regulate the functions of SMAD2 and SMAD3 by phosphorylating a linker region that is not phosphorylated by type I/II receptors [168]. Thus, experimental approaches traditionally thought to address only SMAD-independent signaling (e.g. MAPK inhibitors) have the potential to alter SMAD-dependent actions as well. We used knockdown of SMAD2, SMAD3 or SMAD4 to show that the suppressive effects of activin B on E-cadherin expression are not mediated by canonical SMAD signaling. Interestingly, we have previously demonstrated that both SMAD2 and SMAD3 are required for activin B-induced integrin β 3 up-regulation [149]. Together, our studies suggest that type II endometrial cancer cell migration/invasion involves both MEK-ERK1/2-SNAIL-mediated E-cadherin down-regulation and SMAD2/3-SMAD4-mediated integrin β 3 up-regulation. In addition to regulating cell migration, MEK-ERK1/2 signaling can promote endometrial cancer cell proliferation [169, 170]. However, a recent phase II study of the MEK inhibitor Selumetinib demonstrated only limited single-agent activity in endometrial cancer [171]. These results could be explained by the presence of other oncogenic signaling pathways, such as PI3K/AKT, Wnt/ β -catenin, SMAD, epidermal growth factor receptor/HER2 etc. [172]. In this context, therapeutic approaches

targeting multiple pathways may yield improved activity over single-agent treatments. For example, phase II studies are currently underway to assess the efficacy of MEK inhibition (Trametinib) alone or in combination with AKT inhibition (GSK2141795) in endometrial cancer (NCT01935973). Our results suggest that approaches aimed at inhibiting activin receptor activity could impact multiple downstream pathways and warrant further investigation in type II endometrial cancer. Presently, phase I studies with an ACVR2B Fc fusion protein (STM 434) in combination with liposomal doxorubicin are underway in patients with advanced tumors, including endometrial cancer (NCT02262455).

Our study provides important insights into the SMAD-independent actions of activin B in type II endometrial cancer cells. In summary, we demonstrate that activin B induces the activation of MEK-ERK1/2 signaling which stimulates the production of SNAIL. This up-regulation of SNAIL is required for the down-regulation of E-cadherin which is necessary for activin B-induced cell migration. Therapeutic approaches targeting the molecular mechanisms contributing to invasion/metastasis have the potential to significantly improve the clinical outcomes of patients with type II endometrial cancer.

4.4 Materials and methods

Cell culture

The KLE human endometrial cancer cell line was purchased from the American Type Culture Collection (Manassas, VA). The HEC-50 human endometrial cancer cell line was obtained from the OVCARE Cell Bank (Vancouver, BC). Both cell lines were cultured in DMEM/nutrient mixture F-12 Ham (DMEM/F12; Gibco, Life Technologies, Burlington, ON) supplemented with

100 U/mL penicillin (Gibco, Life Technologies), 100 µg/mL streptomycin (Gibco Life Technologies), and 10% (vol/vol) fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT). Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO₂ in air.

Antibodies and reagents

The following rabbit polyclonal antibodies were obtained from Cell Signaling Technology (Danvers, MA): human SMAD4 (#9515), human phospho-p44/42 MAPK (ERK1/2, Thr202/Tyr204; #9101), rat p44/42 MAPK (ERK1/2; #9102), mouse phospho-AKT (Ser473; #9271), mouse AKT (#9272), human phospho-p38 MAPK (Thr180/Tyr182; #9211), and human p38 MAPK (#9212). The following rabbit monoclonal antibodies were obtained from Cell Signaling Technology: human phospho-SMAD2 (Ser465/467; 138D4), human phospho-SMAD3 (Ser423/425; C25A9), and human SMAD3 (C67H9). The mouse monoclonal antibodies used were: human SMAD2 (L16D3, Cell Signaling Technology), human E-cadherin (#610404, BD Biosciences, Mississauga, ON), human SNAIL (#3895, Cell Signaling Technology) and sea urchin α -tubulin (B-5-1-2, Santa Cruz Biotechnology, Dallas, TX). Horseradish peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were obtained from Bio-Rad Laboratories (Mississauga, ON). SB431542 was purchased from Sigma-Aldrich (Oakville, ON). U0126 was obtained from Calbiochem (San Diego, CA). Recombinant human activin B was obtained from R&D Systems (Minneapolis, MN).

Transwell migration assay

Cell culture inserts (24-well, pore size 8 µm; BD Biosciences) were seeded with 1×10^5 cells in 250 µL of medium supplemented with 0.1% FBS. Medium with 10% FBS (750 µL) was added

to the lower chamber and served as a chemotactic agent. After incubation for 24 h, non-migrating cells were removed from the upper side of the membrane, and the cells on the lower side of the membrane were fixed with cold methanol and air dried. Cells were stained with Crystal Violet and counted using a light microscope. Each individual experiment was performed with triplicate inserts and five microscopic fields were counted per insert.

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

Total RNA was extracted using TRIzol reagent (Invitrogen, Life Technologies, Burlington, ON) in accordance with the manufacturer's instructions. Reverse transcription was performed with 2 µg RNA, random primers and M-MLV reverse transcriptase (Promega, Madison, WI). Each 20 µl SYBR Green RT-qPCR reaction contained 1×SYBR Green PCR Master Mix (Applied Biosystems), 12 ng cDNA and 150 nM of each specific primer. The primers used were: E-cadherin (CDH1), 5'-ACA GCC CCG CCT TAT GAT T-3' (forward) and 5'-TCG GAA CCG CTT CCT TCA-3' (reverse); SNAIL (SNAIL1), 5'-CCC CAA TCG GAA GCC TAA CT-3' (forward) and 5'-GCT GGA AGG TAA ACT CTG GAT TAG A-3' (reverse); SLUG (SNAIL2), 5'-TTC GGA CCC ACA CAT TAC CT-3' (forward) and 5'-GCA GTG AGG GCA AGA AAA AG-3' (reverse); TWIST (TWIST1), 5'-GGA GTC CGC AGT CTT ACG AG-3' (forward) and 5'-TCT GGA GGA CCT GGT AGA GG-3' (reverse); ZEB1, 5'-GCA CCT GAA GAG GAC CAG AG-3' (forward) and 5'-TGC ATC TGG TGT TCC ATT TT-3' (reverse); and GAPDH, 5'-GAG TCA ACG GAT TTG GTC GT-3' (forward) and 5'-GAC AAG CTT CCC GTT CTC AG-3' (reverse). RT-qPCR was performed using an Applied Biosystems 7300 Real-Time PCR System equipped with 96-well optical reaction plates. The specificity of each assay was validated by melting curve analysis and agarose gel electrophoresis of the PCR products. Assay

performance was validated by assessing amplification efficiencies by means of calibration curves, and ensuring that the plot of log input amount versus ΔC_q has a slope with an absolute value <0.1 . At least three separate experiments were performed and each sample was assayed in triplicate. A mean value of the triplicates was used for the determination of relative mRNA levels by the comparative C_q method with GAPDH as the reference gene and using the formula $2^{-\Delta\Delta C_q}$.

Western blot

Cells were lysed in ice cold lysis buffer (Cell Signaling Technology) with added protease inhibitor cocktail (Sigma-Aldrich). Extracts were centrifuged at $20,000\times g$ for 10 min at $4^\circ C$ and supernatant protein concentrations were determined using the DC Protein Assay (Bio-Rad Laboratories). Equal amounts of protein were separated by SDS polyacrylamide gel electrophoresis and transferred onto PVDF membranes. After blocking for 1 h with 5% non-fat dry milk in TBS, the membranes were incubated overnight at $4^\circ C$ with primary antibodies that were diluted 1000-fold in 5% non-fat milk-TBS. Following primary antibody incubation, the membranes were incubated with the appropriate HRP-conjugated secondary antibody. Immunoreactive bands were detected using enhanced chemiluminescent substrate or SuperSignal West Femto chemiluminescent substrate and CL-XPosure film (Thermo Fisher, Ottawa, ON). Membranes were stripped with stripping buffer (50 mM Tris-HCl pH 7.6, 10 mM β -mercaptoethanol, and 1% SDS) at $50^\circ C$ for 30 minutes and reprobed with anti- α -tubulin, anti-ERK1/2, anti-AKT or anti-p38 MAPK as loading controls. Immunoreactive band intensities were quantified by densitometry using Scion Image software (Scion Corp, Frederick, MD) and normalized to those of the relevant loading control.

Small interfering RNA (siRNA) transfection and E-cadherin overexpression

To knock down endogenous SMAD2, SMAD3, SMAD4 and SNAIL, forty percent confluent cells were transfected for 48 h with 20 nM ON-TARGET*plus* SMART pool siRNA targeting human SNAIL, SMAD2, SMAD3 and SMAD4 (Dharmacon, Lafayette, CO) using Lipofectamine RNAiMAX (Invitrogen, Life Technologies). ON-TARGET*plus* Non-targeting pool siRNA (Dharmacon) was used as the control.

To overexpress E-cadherin, eighty percent confluent cells were transfected for 48 h with 1 µg empty vector (pcDNA3.1, Invitrogen, Life Technologies) or vector encoding full-length human E-cadherin (plasmid #45769, Addgene, Cambridge, MA) using Lipofectamine LTX (Life Technologies).

Statistical analysis

Results are presented as the mean \pm SEM of at least three independent experiments. For experiments involving only two groups, results were analyzed by Two-Sample t-test assuming unequal variances using Excel. Multiple group comparisons were analyzed by one-way ANOVA followed by Student-Newman-Keuls test using PRISM software (GraphPad Software). Means were considered significantly different if $P < 0.05$ and are indicated by different letters.

A

Altered in 30 (57%) of 53 endometrial carcinomas with serous histology



B

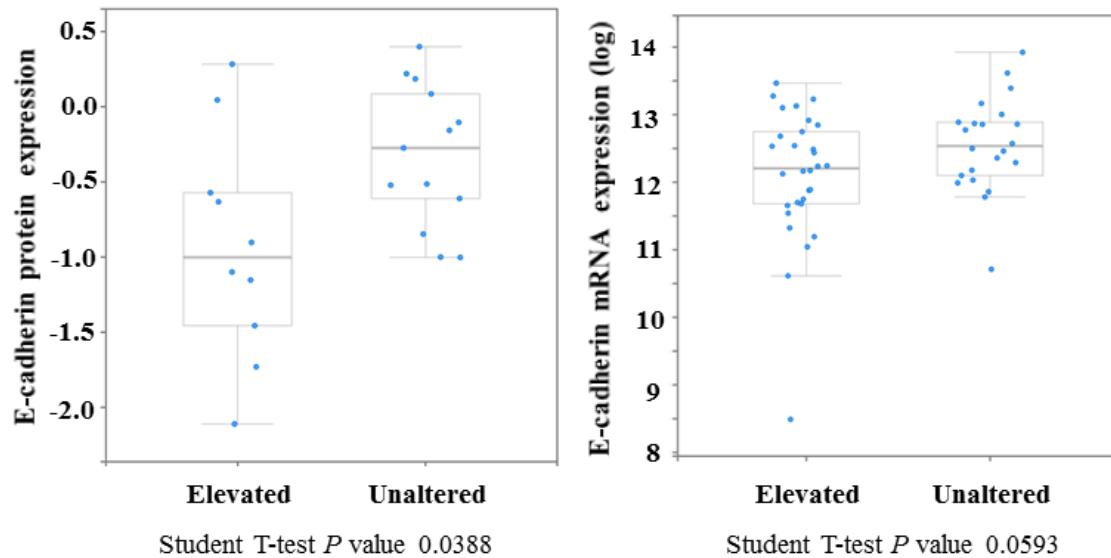


Figure 4. 1 Enhanced activin B signaling may contribute to the down-regulation of E-cadherin in serous endometrial cancers.

The cBioPortal for Cancer Genomics was used to query serous endometrial carcinomas from The Cancer Genome Atlas (n=53) for up-regulation (upper quartile) of at least one component of the activin B ligand-receptor gene set, including inhibin β B (INHBB), type I receptor ACVR1B, and type II receptors ACVR2A and ACVR2B. (A) OncoPrint showing cases with elevated mRNA levels of INHBB, ACVR1B, ACVR2A or ACVR2B across all 53 serous endometrial carcinomas. (B) Enrichment analysis comparing E-cadherin protein (*left*) and mRNA (*right*) levels between unaltered samples and those with INHBB, ACVR1B, ACVR2A or ACVR2B mRNA levels in the upper quartile (elevated). Expression levels of E-cadherin protein (from

reverse-phase protein array) and mRNA (from RNA-Seq V2 RSEM) are displayed as boxplots with a P value from a Student T-test.

A

Altered in 188 (62%) of 307 endometrial carcinomas with endometrioid histology

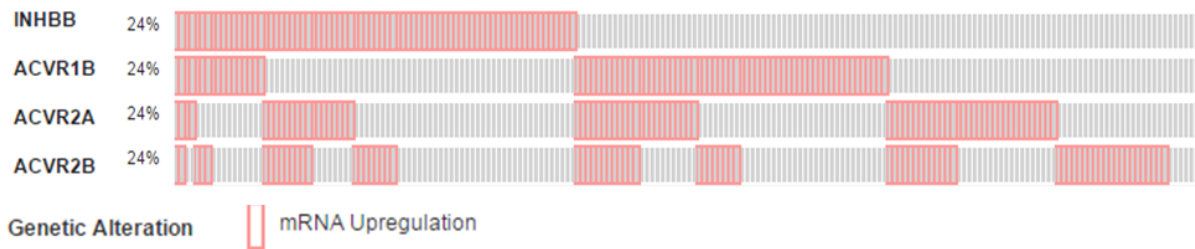
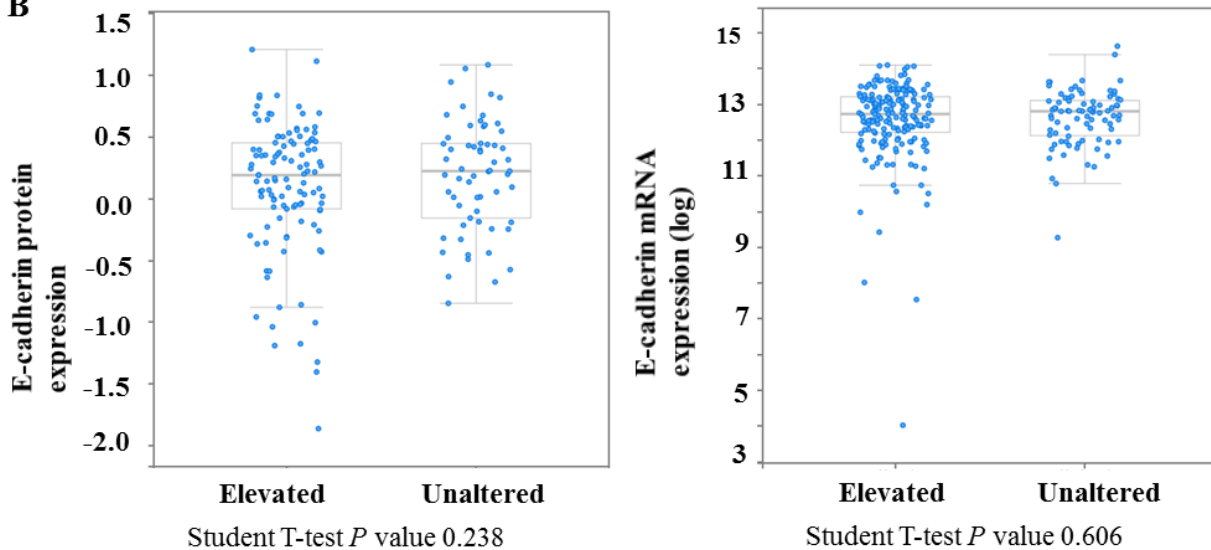
**B**

Figure 4. 2 Enhanced activin B signaling is not associated with the down-regulation of E-cadherin in endometrioid endometrial cancers.

The cBioPortal for Cancer Genomics was used to query endometrioid endometrial carcinomas from The Cancer Genome Atlas (n=307) for up-regulation (upper quartile) of at least one component of the activin B ligand-receptor gene set, including inhibin β B (INHBB), type I receptor ACVR1B, and type II receptors ACVR2A and ACVR2B. (A) OncoPrint showing cases with elevated mRNA levels of INHBB, ACVR1B, ACVR2A or ACVR2B across all 307 endometrioid endometrial carcinomas. (B) Enrichment analysis comparing E-cadherin protein (left) and mRNA (right) levels between unaltered samples and those with INHBB, ACVR1B, ACVR2A or ACVR2B mRNA levels in the upper quartile (elevated). Expression levels of E-

cadherin protein (from reverse-phase protein array) and mRNA (from RNA-Seq V2 RSEM) are displayed as boxplots with a P value from a Student T-test.

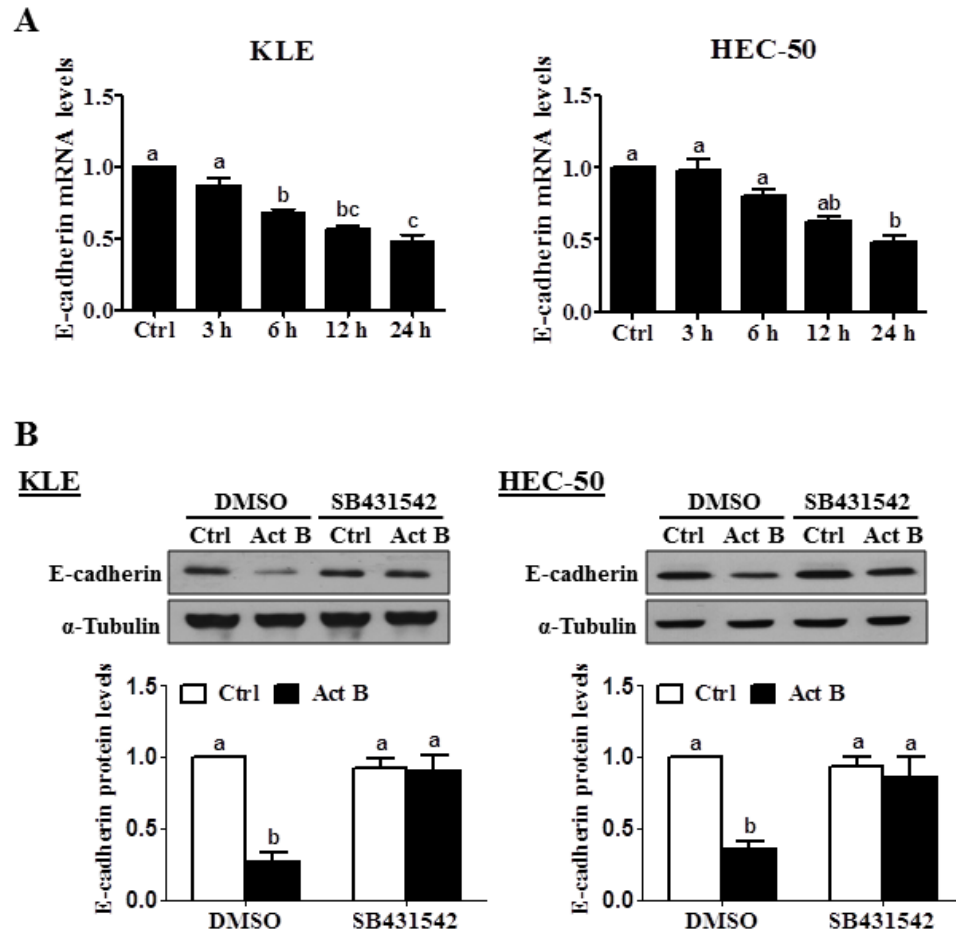


Figure 4. 3 Activin B down-regulates E-cadherin expression in human endometrial cancer cells.

A, KLE and HEC-50 cells were treated for varying times without (Ctrl; time-matched controls displayed as single bar) or with 50 ng/mL activin B and E-cadherin mRNA levels were examined by RT-qPCR. B, KLE and HEC-50 cells were pre-treated with vehicle control (DMSO) or SB431542 (10 μ M) for 1 h and then treated with or without 50 ng/mL activin B (Act B) for 24 h. Protein levels of E-cadherin were examined by Western blot. Results are expressed as the mean \pm SEM of at least three independent experiments and values without common letters are significantly different ($P < 0.05$).

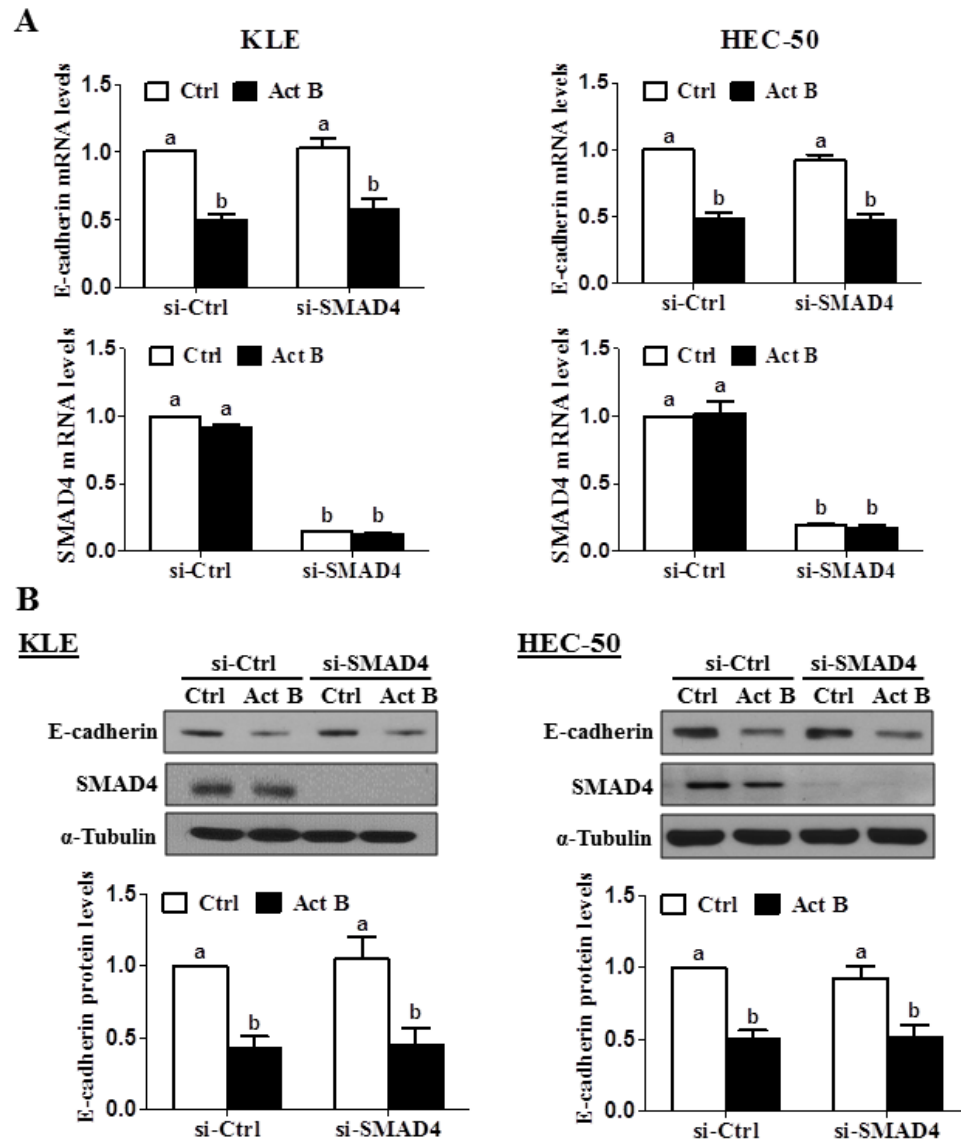


Figure 4. 4 SMAD4 is not required for the down-regulation of E-cadherin by activin B.

KLE and HEC-50 cells were transfected for 48 h with 20 nM control siRNA (si-Ctrl) or SMAD4 siRNA (si-SMAD4) and then treated without (Ctrl) or with 50 ng/mL activin B (Act B) for 24 h. E-cadherin and SMAD4 mRNA (A) and protein (B) levels were measured by RT-qPCR and Western blot, respectively. Results are expressed as the mean \pm SEM of at least three independent experiments and values without common letters are significantly different ($P < 0.05$).

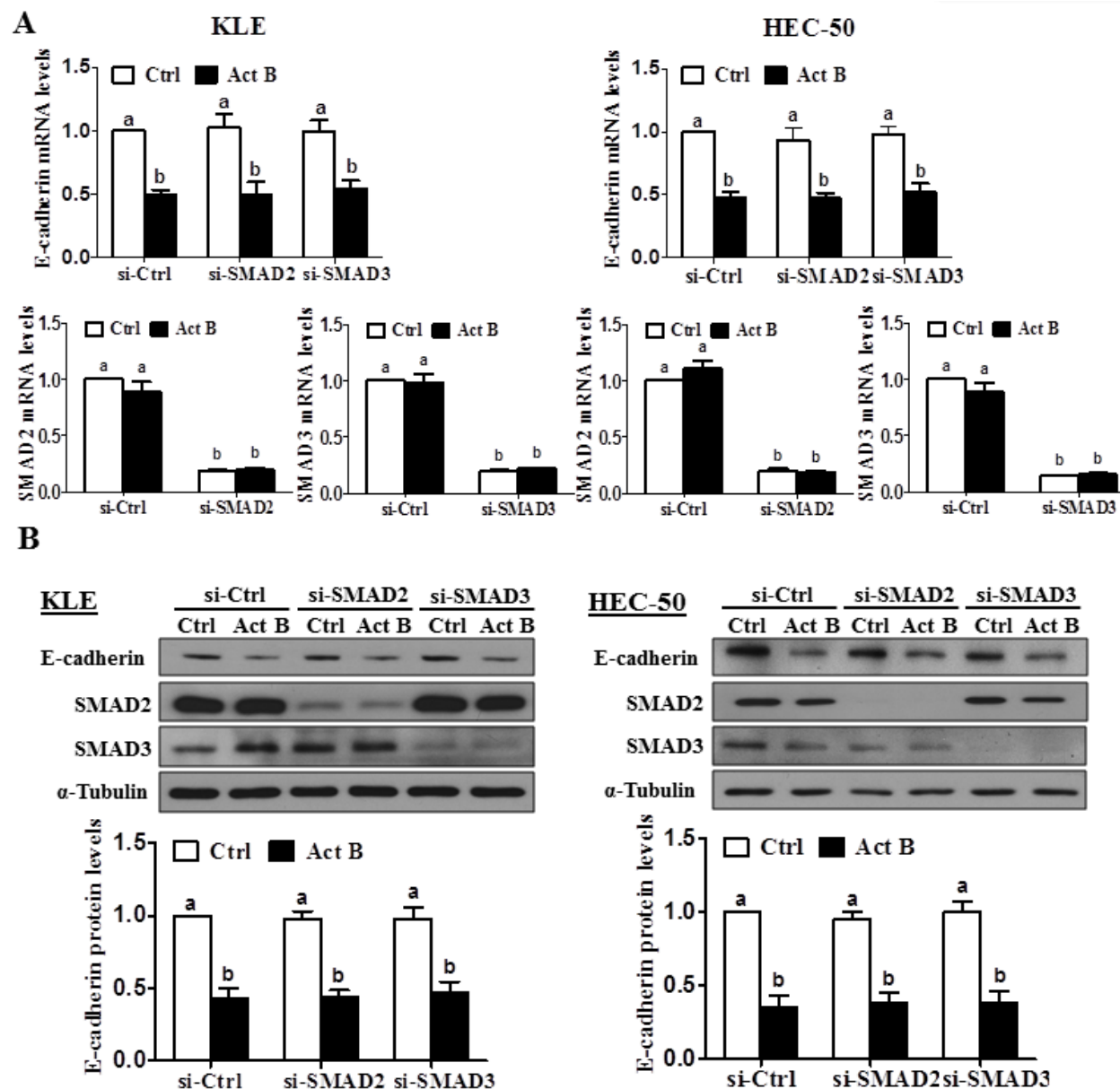


Figure 4. 5 SMAD2 and SMAD3 are not required for activin B-induced down-regulation of E-cadherin.

KLE and HEC-50 cells were transfected for 48 h with 20 nM control siRNA (si-Ctrl), SMAD2 siRNA (si-SMAD2) or SMAD3 siRNA (si-SMAD3) and then treated without (Ctrl) or with 50 ng/mL activin B (Act B) for 24 h. E-cadherin, SMAD2 and SMAD3 mRNA (A) and protein (B) levels were measured by RT-qPCR and Western blot, respectively. Results are expressed as the

mean \pm SEM of at least three independent experiments and values without common letters are significantly different ($P < 0.05$).

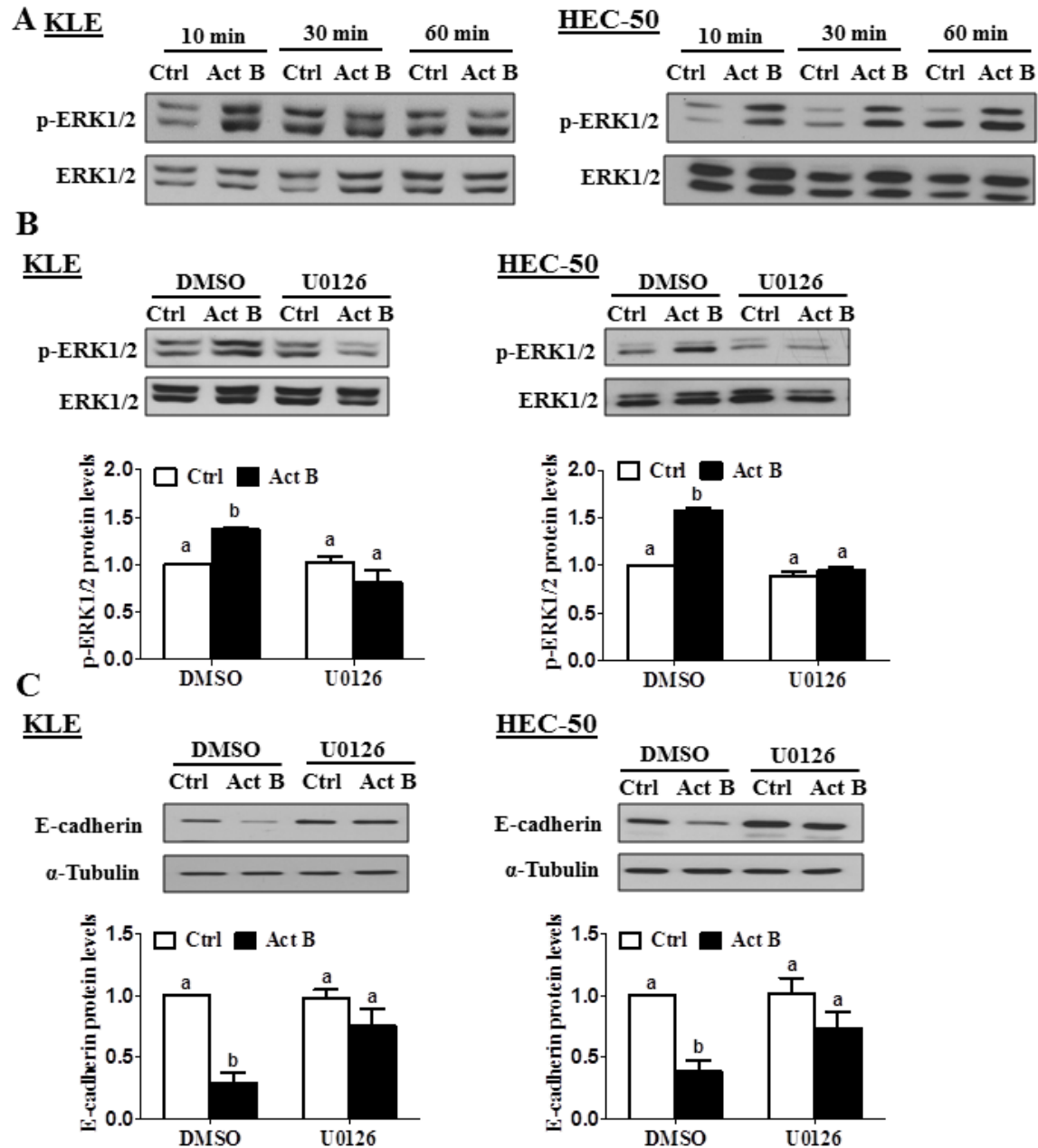


Figure 4. 6 MEK-ERK1/2 signaling is required for the down-regulation of E-cadherin by activin B.

A, KLE and HEC-50 cells were treated without (Ctrl) or with 50 ng/mL activin B (Act B) for 10, 30 or 60 min and Western blot was used to examine the levels of phosphorylated ERK1/2 (p-ERK1/2) in relation to total levels of ERK1/2. B, KLE and HEC-50 cells were pre-treated with vehicle control (DMSO) or U0126 (10 μ M) for 1 h and then treated with or without 50 ng/mL

activin B for 10 min. Western blot was used to measure ERK1/2 phosphorylation. C, KLE and HEC-50 cells were pre-treated with or without U0126 (10 μ M) for 1 h and then treated with or without 50 ng/mL activin B for 24 h. Protein levels of E-cadherin were examined by Western blot. Results are expressed as the mean \pm SEM of at least three independent experiments and values without common letters are significantly different ($P < 0.05$).

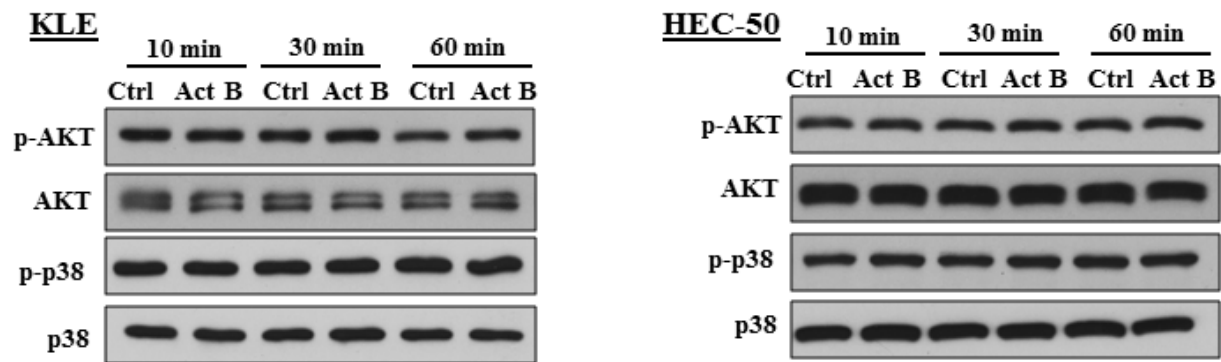


Figure 4. 7 Effects of activin B on AKT and p38 MAPK phosphorylation in endometrial cancer cells.

KLE and HEC-50 cells were treated without (Ctrl) or with 50 ng/mL activin B (Act B) for 10, 30 or 60 min and Western blot was used to examine the levels of phosphorylated AKT (p-AKT) and p38 MAPK (p-p38) in relation to their total levels (AKT and p38, respectively).

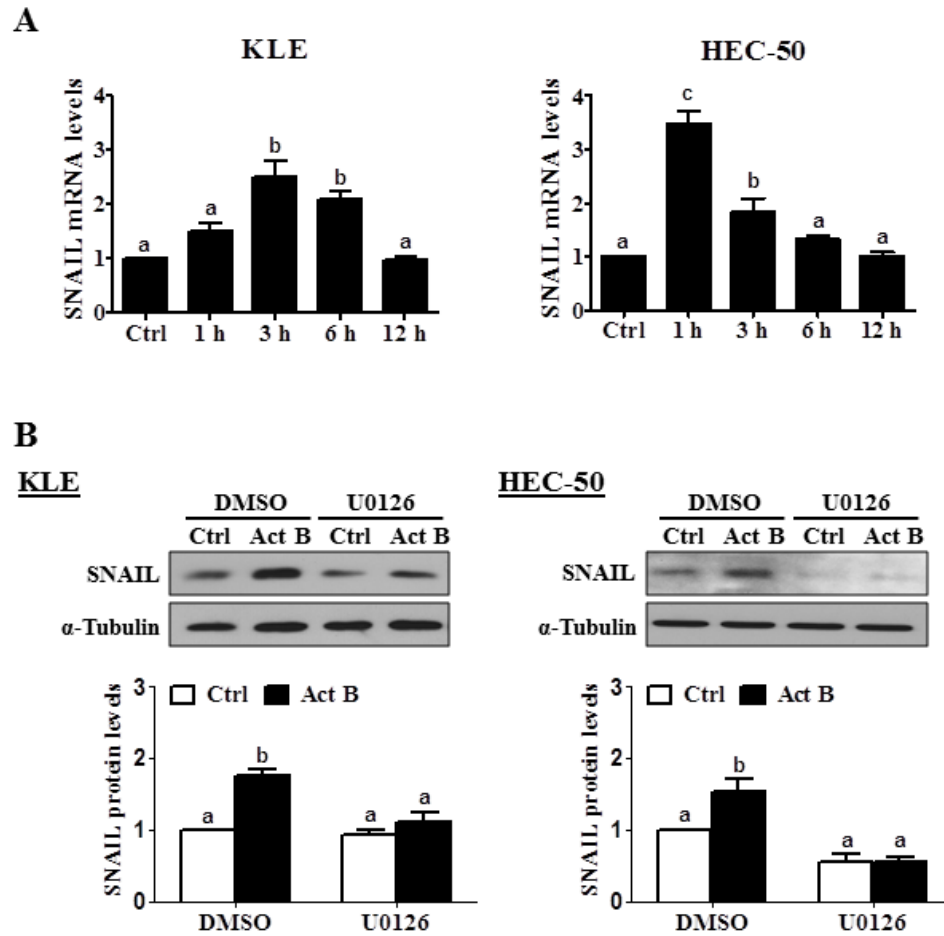


Figure 4. 8 Activin B up-regulates SNAIL via MEK-ERK1/2 signaling.

A, KLE and HEC-50 cells were treated for varying times without (Ctrl; time-matched controls displayed as single bar) or with 50 ng/mL activin B and SNAIL mRNA levels were examined by RT-qPCR. B, KLE and HEC-50 cells were pre-treated with vehicle control (DMSO) or U0126 (10 μ M) for 1 h and then treated with or without 50 ng/mL activin B (Act B) for 3 h. Protein levels of SNAIL were examined by Western blot. Results are expressed as the mean \pm SEM of at least three independent experiments and values without common letters are significantly different ($P < 0.05$).

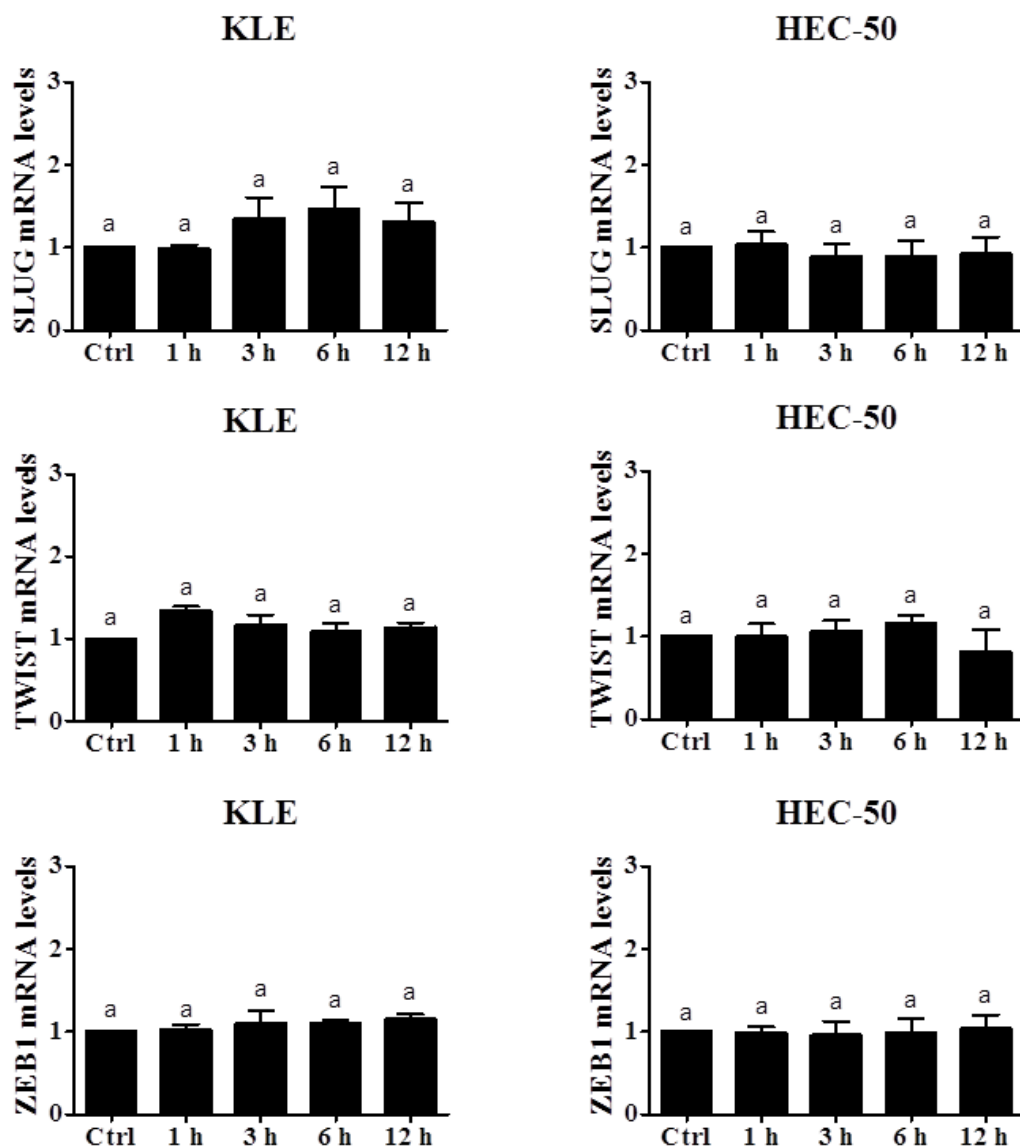


Figure 4. 9 Activin B does not alter the mRNA levels of SLUG, TWIST and ZEB1.

KLE and HEC-50 cells were treated for varying times without (Ctrl; time-matched controls displayed as single bar) or with 50 ng/mL activin B and SLUG (A), TWIST (B) and ZEB1 (C) mRNA levels were examined by RT-qPCR. Results are expressed as the mean \pm SEM of at least three independent experiments and there were no significant differences between any of the groups.

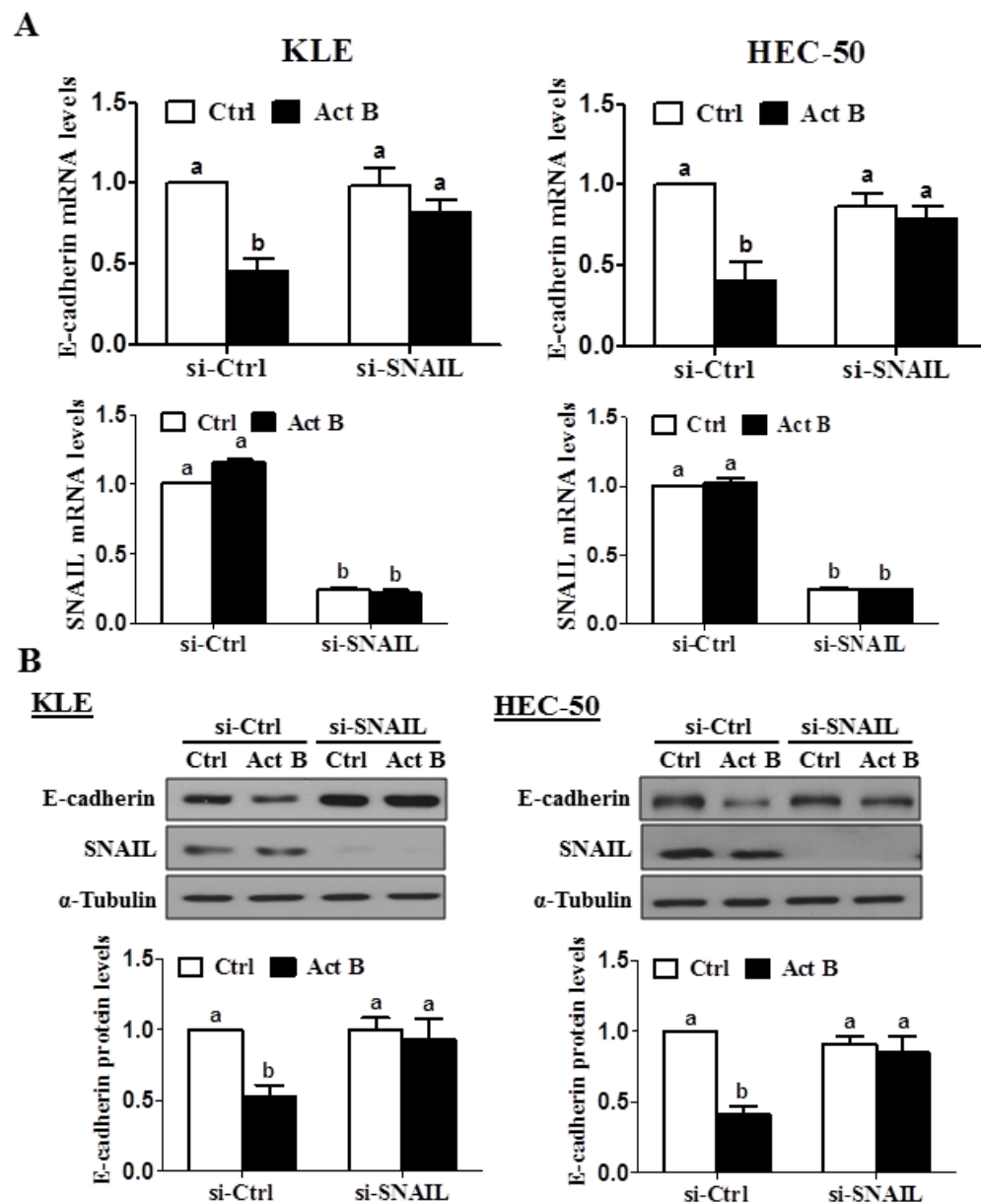


Figure 4. 10 SNAIL is required for the down-regulation of E-cadherin by activin B.

KLE and HEC-50 cells were transfected for 48 h with 20 nM control siRNA (si-Ctrl) or SNAIL siRNA (si-SNAIL) and then treated without (Ctrl) or with 50 ng/mL activin B (Act B) for 24 h. E-cadherin and SNAIL mRNA (A) and protein (B) levels were measured by RT-qPCR and Western blot, respectively. Results are expressed as the mean \pm SEM of at least three

independent experiments and values without common letters are significantly different ($P < 0.05$).

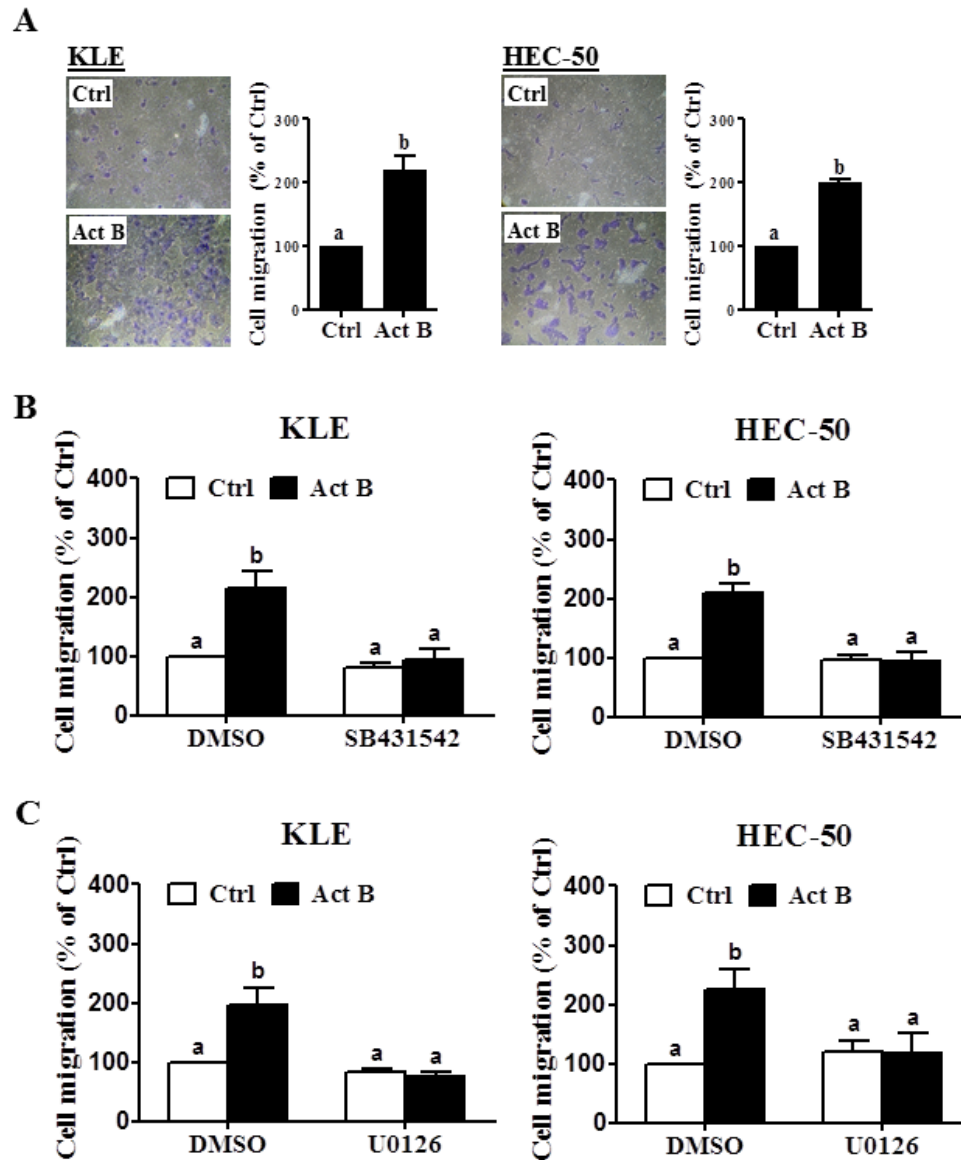


Figure 4. 11 MEK-ERK1/2 signaling is required for activin B-induced cell migration.

A, KLE and HEC-50 cells were treated without (Ctrl) or with 50 ng/mL activin B (Act B) for 24 h and then seeded in transwell inserts for migration assay. For each cell line, *left panels* show representative photomicrographs of migrating cells, while *right panels* show summarized quantitative results. B and C, Migration assays were performed with KLE and HEC-50 cells following pre-treatment with vehicle control (DMSO), SB431542 (10 μ M) (B) or U0126 (10 μ M) (C) for 1 h prior to treatment with or without 50 ng/mL activin B for 24 h. Results are

expressed as the mean \pm SEM of at least three independent experiments and values without common letters are significantly different ($P < 0.05$).

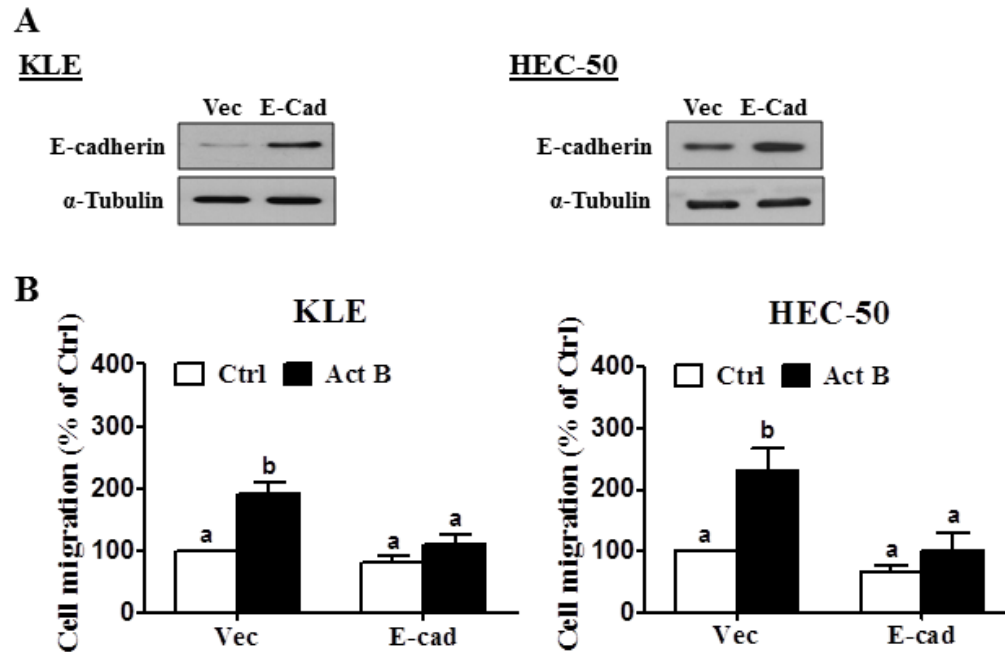


Figure 4. 12 Forced-expression of E-cadherin inhibits activin B-induced cell migration.

A, KLE and HEC-50 cells were transfected for 48 h with control vector (Vec) or vector encoding full-length human E-cadherin (E-cad). Protein levels of E-cadherin were examined by Western blot. B, Transwell migration assays were performed with KLE and HEC-50 cells following transfection for 48 h with control vector or E-cadherin vector prior to treatment without (Ctrl) or with 50 ng/mL activin B for 24 h. Results are expressed as the mean \pm SEM of at least three independent experiments and values without common letters are significantly different ($P < 0.05$).

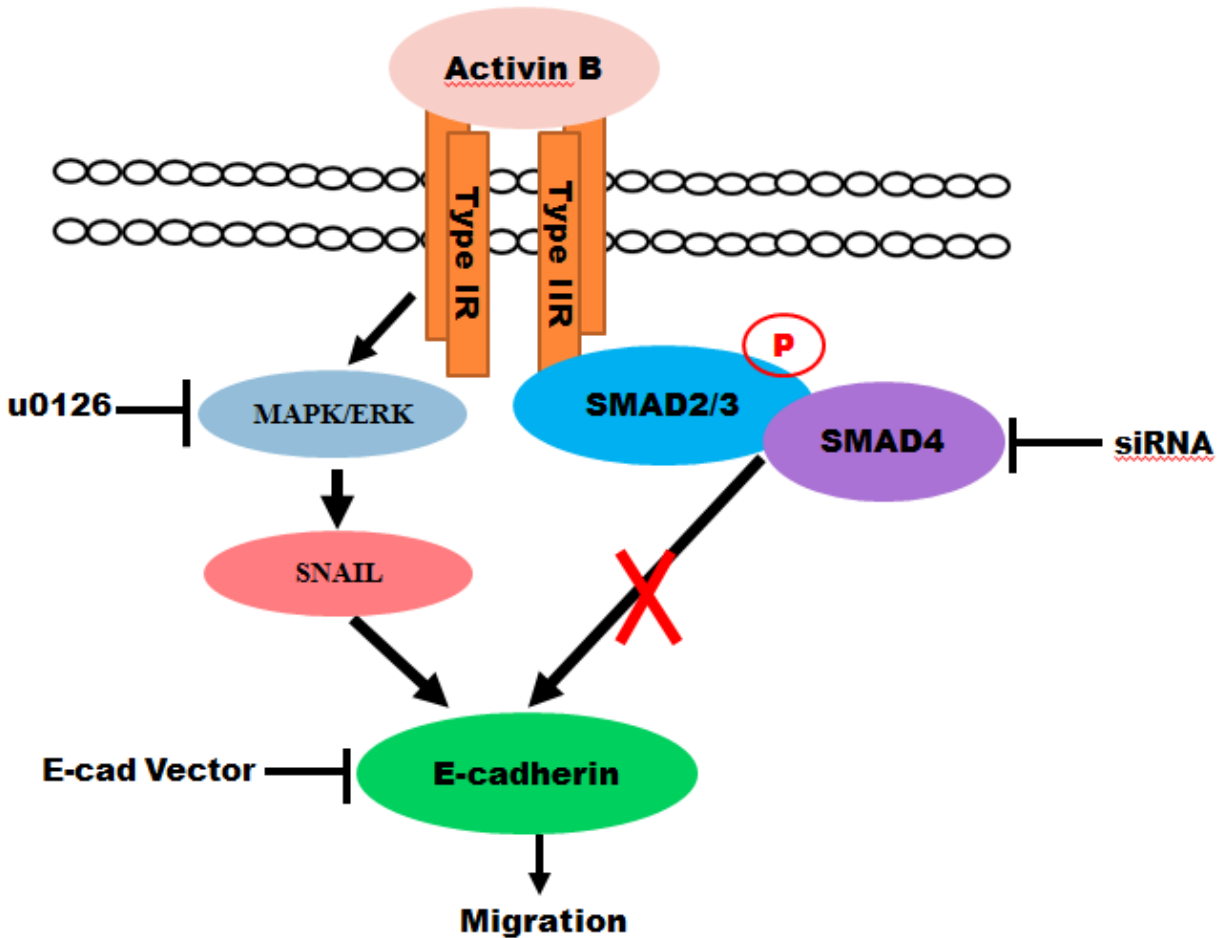


Figure 4. 13 A schematic illustration of Chapter 4 data

Treatment of HEC-1B and KLE cells with Activin B decreased E-cadherin expression (Figure 4.3) while generated comparable increases in ERK phosphorylation (Figure 4.6) and SNAIL expression (Figure 4.8). Knockdown of SMAD2, 3, or 4 (Figure 4.4 and 4.5) failed to reduce the effects of activin B on E-cadherin. Co-treatment with the MEK-ERK1/2 inhibitor U0126 abolished activin B induced down-regulation of E-cadherin, up-regulation of SNAIL and cell migration (Figure 4.6, 4.8 and 4.11). Importantly, activin B induced cell migration was attenuated by E-cadherin vector mediated overexpression of E-cadherin (Figure 4.13).

Chapter 5: TGF β 1 induces human endometrial cancer cell adhesion and migration by up-regulating integrin β 3 and α v via SMAD-independent MEK-ERK1/2 signaling

5.1 Introduction

Endometrial cancer is the second most lethal gynecological tract malignancy in North America. With the development of basic science and translational medicine, the mortality of many cancers have been effectively reduced. However, the number of endometrial cancer related deaths has been growing [8], which may be explained by the increased incidence of advanced-stage tumor and high risk histologies [88]. Endometrial cancer is traditionally classified into type I (mostly low-grade endometrioid cancer) and type II (predominantly serous cancer) based on clinicopathological features. While type I cancer is low grade, usually confined to the uterus at diagnosis, type II endometrial cancer often behaves aggressively characterized by myometrial invasion and extrauterine metastasis [83]. From a clinical perspective, the extent of myometrial invasion and metastasis is the most important prognostic factor in endometrial cancer [89]. Consistently, while type I endometrioid endometrial cancer shows excellent cure rate, type II serous endometrial cancer accounts for ~40% of endometrial cancer deaths, despite accounting for only ~10% of all endometrial cancer [83]. However, the key molecular events which trigger endometrial cancer invasion and dissemination still remain delineated [90].

TGF β 1 belongs to TGF β superfamily. They bind to type II transmembrane serine–threonine kinase receptor (TGFB β R2), which in turn activates type I receptor (TGFB β R1), leading to the phosphorylation of classical SMAD signaling pathways. They can also signal through SMAD independent pathways such as MAPK pathways [164]. TGF β signaling is essential for critical

biological processes including cell proliferation, differentiation, apoptosis and extracellular matrix remodeling. Although TGF β 1 inhibits the proliferation of many types of epithelial cells, it tends to promote the invasiveness of various tumors [38] and is associated with poorer patient outcome [93]. In uterus, TGF β 1 plays critical roles in the physiology and pathology of endometrium such as decidualization, implantation, placental development, extracellular matrix remodeling or endometriosis [5]. Importantly, growing evidences suggest that TGF β signalings are closely associated with the neoplastic transformation and invasion of endometrial cancer. There is a statistically significant gradual increase in the immune-staining of all three TGF β s progressing from the normal proliferative endometrium to simple hyperplasia and on to complex hyperplasia [37]. Consistently, TGF β 1 plasma level is greater in Stage-Ib and Stage-Ic patients than in Stage-Ia patients [38]. TGFBR2 protein level estimated by ELISA is higher in endometrial cancer compared to normal endometrial tissues [39]. Significantly elevated protein level of TGFBR2 was also noted in endometrial cancer with myometrial invasion compared to those non-infiltrating tumors [39, 40].

While SMAD proteins always act as tumor suppressors, deregulated SMAD signaling was frequently associated with tumor progression [93]. In endometrial cancer, significantly less level of phosphorylated SMAD2 was expressed in tumor tissues than in normal tissues [173]. Also, decreased mRNA levels of SMAD2 and SMAD4 were observed in the infiltrating myometrial uterine tumors compared with non-infiltrating tumors. Interestingly, SMAD4 protein level in the cytoplasmic fraction was increasing as the tumor progresses. Significantly higher protein level of SMAD4 was discovered in the cytoplasmic fractions of infiltrating tumors compared with those restricted to the endometrium, and of poorly differentiated (G3) endometrial cancer compared

with moderately (G2) and well-differentiated endometrial cancer (G1) [40]. Therefore, TGF β 1-SMAD signaling may be deregulated in endometrial cancer which may help switch TGF β 1 from a tumor suppressor to a tumor promoter to enhance the migration capacity of endometrial cancer. Indeed, gene expression profiling of high-risk recurrence endometrial cancer identified TGF β 1 as the core factor in the initiation of tumor invasion. Consistently, in vitro experiments showed that TGF β 1 can promote the invasiveness of KLE, HEC-1A and RL95-2 endometrial cancer cells [38, 41]. While much has been written on the expression and role of TGF β 1 signaling in endometrial cancer, there is little information on the mechanisms of TGF β 1 that governs the migration, the initial stage of metastasis and dissemination. Clarifying the molecular mechanisms underlying TGF β 1 induced migration may give rise to attractive strategy to inhibit tumor spread and improve the outcome of advanced-stage endometrial cancer.

In the present study, we examined the effects and mechanisms of TGF β 1 on endometrial cancer cell adhesion and migration. We demonstrate that TGF β 1 promotes KLE and HEC-1B cell adhesion to vitronectin and migration by the MEK-ERK1/2 dependent up-regulation of integrin β 3 and α v. Our findings suggest that TGF β 1 signaling could promote the metastasis of type II endometrial cancers, thereby contributing to poor patient survival.

5.2 Results

TGF β 1 increases endometrial cancer cell adhesion and migration

TGF β 1 has been found to affect the expression of integrins and the subsequent cell adhesion and migration [174]. Therefore we investigated the biological functions of TGF β 1 on adhesion and migration in two type II endometrial cancer cell lines (KLE and HEC-1B). We first

examined the effects of TGF β 1 on cell adhesion to different extracellular matrix proteins. As shown in Figure 5.1A, KLE cell adhesiveness was increased in vitronectin-, fibronectin- or collagen IV-coated tissue culture plates compared to uncoated plates. Interestingly, treatment with 10 ng/mL TGF β 1 significantly enhanced the adhesion of KLE cells to vitronectin and fibronectin, but did not affect adhesion to collagen IV or uncoated plates (Figure 5.1A). However, TGF β 1 treatment increased the adhesion of HEC-1B cells only to vitronectin (Figure 5.1A). We also performed Transwell migration assays. As shown in Figure 5.1B, both KLE and HEC-1B exhibited basal levels of cell motility. Importantly, treatment with 10 ng/ml TGF β 1 significantly increased cell migration in both cell lines.

TGF β 1 up-regulates both integrin β 3 and integrin α v

Given that TGF β 1 only specifically enhanced cell adhesion to vitronectin in both endometrial cancer cell lines, we next examined its effects on the expression of integrin α v β 3, a well-known receptor for vitronectin [175]. As shown in Figure 5.2A, treatment with TGF β 1 for different periods of time significantly up-regulated both integrin β 3 and integrin α v mRNA levels and the effect was still observed after 24 h of treatment (Figure 5.2A). Western blot analysis was used to confirm the similar stimulatory effects of TGF β 1 on integrin β 3 and integrin α v protein levels, and to show that they could be abolished by pre-treatment with SB431542 (Figure 5.2B), an inhibitor of activin/TGF β type I receptors.

Our results are consistent with the enrichment analysis of endometrial cancers with serous histology from TCGA ([11]; n=53), which compared integrin β 3 or α v mRNA levels between unaltered samples and those with elevation of at least one component of the TGF β ligand-

receptor gene set, including TGF β , type I receptor TGFBR1, type II receptor TGFBR2. Serous tumors with TGF β , TGFBR1, and TGFBR2 mRNA levels in the upper quartile displayed significantly elevated levels of integrin β 3 mRNA (P=0.0024) and integrin α v mRNA (P=0.0196) (Figure 5.3). We also performed mutual exclusivity analysis among TGF β ligand-receptor gene set and integrin β 3/ α v. In serous tumors with TGF β ligand-receptor gene set and integrin β 3/ α v mRNA levels in the upper quartile, there is a significant co-occurrence tendency between TGF β 1 and integrin β 3 (ITGB3), TGFBR2 and integrin β 3 (ITGB3), TGFBR2 and integrin α v (ITGAV). Similarly, in the co-expression analysis, TGF β 1 tends to be co-expressed with integrin β 3 (ITGB3). These findings suggest enhanced TGF β signaling contributes to the elevation of integrin α v β 3 in type II serous endometrial cancer cells.

SMAD signaling is not required for TGF β -induced up-regulation of integrin β 3 or α v

To examine the activation of canonical SMAD2/SMAD3 signaling, KLE and HEC-1B cells were treated with TGF β 1 and Western blot was used to measure the levels of phosphorylated SMAD2 and SMAD3 in relation to their total levels. As shown in Figure 5.4A, treatment with TGF β 1 for 30 or 60 minutes induced the phosphorylation of SMAD2 and SMAD3 in both cell lines. Next, we used pre-treatment with siRNA targeting common SMAD4 to investigate the involvement of SMAD signaling in the up-regulation of integrin β 3 or α v by TGF β 1. As shown in Figure 5.4B, despite reducing SMAD4 mRNA levels by more than 80%, pre-treatment with SMAD4 siRNA did not alter the stimulatory effects of TGF β 1 on integrin β 3 or α v mRNA levels in either cell line. Similarly, Western blot analysis showed that the stimulatory effects of TGF β 1 on integrin β 3 or α v protein levels were not affected by SMAD4 knockdown (Figure 5.4C).

MEK-ERK1/2 signaling is required for the up-regulation of integrin $\beta 3$ or αv by TGF β 1

Apart from canonical SMAD signaling, MAPK pathways are suggested to have a particularly complicated and intimate inter-relationship with TGF β systems in tumorigenesis [176]. More importantly, MAPK and TGF β 1 pathways are simultaneously identified as key factors of high-risk recurrence in a bioinformatics analysis of a sizable cohort of endometrial carcinomas [41]. To examine the activation of MAPK pathways, we treated KLE and HEC-1B cells with TGF β 1 and used Western blot to measure the level of phosphorylated ERK1/2 in relation to its total levels. Treatment with TGF β 1 induced the phosphorylation of ERK1/2 in both cell lines after 10 min, but the effect was lost after 1 h (Figure 5.5A). We then used the MEK inhibitor U0126 to determine whether MEK-ERK1/2 signaling is required for the effects of TGF β 1 on integrin $\beta 3$ or αv in KLE and HEC-1B cells. Pre-treatment with U0126 inhibited both the induction of ERK1/2 phosphorylation (Figure 5.5B) and the up-regulation of integrin $\beta 3$ or αv (Figure 5.5C) by TGF β 1.

Up-regulation of integrin $\beta 3$ or αv is required for TGF β -induced cell adhesion and migration

The effects of TGF β 1 on cell adhesion and migration were abolished by inhibition of MEK-ERK1/2 signaling (U0126; Figure 5.6A and 5.6B). Next, we used pre-treatment with siRNA targeting integrin $\beta 3$ or αv to investigate their specific roles in TGF β -induced cell adhesion and migration. Western blot analysis confirmed the decreased expression of integrin $\beta 3$ or αv in KLE and HEC-1B cells transfected with specific siRNA (Figure 5.7A). Importantly, adhesion and migration assay results showed that siRNA-knockdown of integrin $\beta 3$ or αv attenuated TGF β -increased cell adhesion and migration in both KLE and HEC-1B cells (Figure 5.7 and 5.8). To

double confirm the role of integrin $\alpha v \beta 3$ heterodimer in TGF β -induced cell adhesion and migration, cells were pre-treated with 10 $\mu\text{g/mL}$ anti-integrin $\alpha v \beta 3$ antibody LM609 before functional assays. Similarly, LM609 completely abolished TGF β -induced cell adhesion and migration (Figure 5.7D and 5.7C).

5.3 Discussion

The close relationships between αv -integrins and TGF β signaling have been identified over the past few years. These include the requirement of $\alpha v \beta 6$ for up-regulation of MMP2 by TGF $\beta 1$ in prostate cancer [177], augmented capability of TGF $\beta 1$ to induce EMT via TGF $\beta 1$ up-regulated integrin $\beta 3$ in mammary epithelial cells [178], and the necessity of $\beta 3$ in the stimulating effects of TGF $\beta 1$ /H $_2$ O $_2$ /HOCl on invasive capacity of hepatocellular carcinoma cells [179]. We demonstrate TGF $\beta 1$ treatment of endometrial cancer cells up-regulates the mRNA and protein levels of both integrin αv and $\beta 3$. These cellular effects are consistent with our TCGA analysis showing up-regulated integrin αv and $\beta 3$ mRNA levels in serous endometrial cancers with elevation of at least one component of the TGF $\beta 1$ ligand-receptor gene set and the tendency towards co-occurrence between TGF $\beta 1$ and integrin $\beta 3$, TGFBR2 and integrin $\beta 3$, TGFBR2 and integrin αv . Integrin $\beta 3$ forms only two α - β heterodimers, $\alpha v \beta 3$ and $\alpha \text{IIb} \beta 3$, both of which are receptors for vitronectin [124]. However, integrin αIIb mRNA levels are nearly undetectable in HEC-1B and KLE cells (data not shown). Therefore, that the knockdown of integrin $\beta 3$ decreased TGF β -induced cell migration and adhesion to vitronectin is likely mediated by integrin $\alpha v \beta 3$. These findings suggest the relationship between TGF β signaling and integrin $\alpha v \beta 3$ may contribute to the aggressive behavior of type II endometrial cancer. Whereas TGF β regulates integrin mediated cell migration and adhesion by altering the expression of integrins,

accumulating evidences suggest that αv integrins can in turn stimulate TGF β signaling by activating the latent form of TGF β , interacting with TGF β receptors, potentiating its downstream signaling, and affecting the expression of TGF β signaling pathway components [180]. Whether there is a crosstalk between integrin $\alpha v\beta 3$ and TGF β in our endometrial cancer cell models requires further studies. But our study suggests that approaches aimed at inhibiting TGF β ligand or receptor activity could impact cancer cell adhesion and metastasis and warrant further investigation in type II endometrial cancer. Currently, many TGF β signaling antagonists are under development for treating various cancers at both the pre-clinical and clinical stages [181]. Importantly, blocking TGF β signaling by ectopic expression of a dominant-negative TGF β type II receptor (DNRII) in endometrial cancer cell line HEC-1A greatly decreased its migration ability in vitro and in mouse models, indicating the potential utility of TGF β receptor inhibitors for the treatment of advanced endometrial cancer [182]. However, TGF $\beta 1$ plays a predominant role in many normal physiological functions, thus long term inhibition of this pathway may lead to harmful off-target effects. To this respect, integrin $\alpha v\beta 3$ can be an attractive therapeutic target to interfere with TGF β signaling in cancer progression. Integrin $\alpha v\beta 3$ was the first integrin observed to be deregulated in a malignant disease, melanoma. Later it was found to be elevated in a wide variety of tumors, and thus became the hotspot therapeutic target. Overall, the safety profiles of systemic long term use of $\alpha v\beta 3$ blockers are favorable. More importantly, cilengitide, an inhibitor of both $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins, has shown great potential in patients with late-stage glioblastoma by extending patient survival with minimal side effects. Other integrin $\alpha v\beta 3$ inhibitors such as etaracizumab (humanized engineered mAb LM609) and CNTO 95 (human αv integrin-specific monoclonal antibody) also showed signs of anti-tumor efficacy in clinical trials. Therefore, they are all been further evaluated [135]. Our study supported the investigation of

integrin $\alpha v \beta 3$ as a novel therapeutic target in type II endometrial cancers by virtue of its ability to promote TGF β -induced cell migration and adhesion.

Currently, far fewer studies have described integrin $\beta 3$ regulation by ERK1/2 signaling. One study demonstrated that pharmacological inhibition of MEK1 by U0126 in human melanoma and pancreatic carcinoma cell lines reduced integrin $\beta 3$ expression. Also, activation of Raf-MEK-ERK pathway in mouse NIH 3T3 cells or human TIME (telomerase immortalized microvascular endothelial) cells induced integrin $\beta 3$ expression on the cell surface. However, in this study, integrin $\beta 3$ was only induced in response to sustained activation of the Raf-MEK-ERK signaling pathway and not in response to the transient activation of this pathway elicited by growth factors and mitogens [183]. Similarly, another study showed that sustained ERK1/2 activity is associated with integrin $\beta 3$ induction [184]. Differing from these studies, we demonstrate that transient activation of ERK signaling by TGF $\beta 1$ which was no longer activated 30 min after treatment can also induce the expression of integrin $\beta 3$. Interestingly, in our study the induction of integrin $\beta 3$ was accompanied by a concomitant increase of integrin αv which usually remains unaltered due to its abundant expression. This is consistent with a study in hepatocellular carcinoma cells where ERK1/2 signaling up-regulated integrin αv expression by promoting SP-1 phosphorylation [185]. In fact, the potential binding sites for SP-1, Ets and GATA transcriptional factors have been found in the promoter region of integrin αv or $\beta 3$ [186-189]. Although these transcription factors have been shown to be regulated by MEK-ERK1/2 signaling in various cell types [190-193], further analysis of the promoter is required to confirm a role for these transcription factors in the control of integrin αv or $\beta 3$ expression in endometrial cancer cells. Whereas TGF $\beta 1$ promotes the expression of integrin αv or $\beta 3$ via ERK1/2 pathway rather than SMAD signaling

in our current study, activin B up-regulates integrin $\beta 3$ via SMAD rather than ERK1/2 pathway in our previous study [149]. Given that TGF β s and activins utilize distinct sets of type I and type II receptors, they may associate with different downstream molecular mediators to regulate integrins. Another explanation might be that there is a particularly intimate inter-relationship between the TGF β system and Ras/MAPK pathways in tumorigenesis [176]. Activation of ERK1/2 pathway antagonizes the SMAD pathway and diverted the TGF β 1 response towards a pro-oncogenic outcome [164].

Extensive literatures have demonstrated that alterations in the expression of integrin subunits can affect the metastatic and invasive properties of cancer cells [194, 195]. For examples, the expression of integrin $\alpha v \beta 3$ is closely associated with the acquisition of metastatic behavior of melanoma and glioblastoma cells [183]. However, there is limited information regarding the expression and function of integrin $\alpha v \beta 3$ in endometrial cancer. One study showed that integrin αv is strongly positive in 100% of primary serous endometrial cancer cells while integrin $\beta 3$ is expressed in 37.5% of cells [196]. This is consistent with our study where integrin αv and $\beta 3$ are strongly expressed in HEC-1B and KLE type II endometrial cancer cell lines and the mRNA level of integrin αv is at least 10 times higher than that of integrin $\beta 3$ (data not shown). High expression of $\alpha v \beta 3$ integrin appears frequently in biologically aggressive tumors and promotes migration and adhesion [126-129]. Regarding endometrial cancer, blocking integrin αv by Intetumumab significantly inhibits serous endometrial cancer cell migration and adhesion to vitronectin [196]. Similarly, we demonstrate blocking of integrin $\alpha v \beta 3$ by siRNA or specific antibody significantly abolished TGF β induced cell migration and adhesion to vitronectin. Considering molecular therapies targeting $\alpha v \beta 3$ have achieved promising results such as disease

stabilization in advanced solid tumors [132, 133] and extended survival in high grade glioma [135], it's reasonable to speculate the potential role of integrin $\alpha v \beta 3$ as a novel therapeutic target in type II endometrial cancer. Interestingly, the effect on the reduction of adhesion and migration is much more obvious in the cells with siRNA knockdown of integrin αv than those with knockdown of integrin $\beta 3$. That maybe because the αv subunit can associate with many different β -subunits such as $\beta 1$, $\beta 3$, $\beta 5$, $\beta 6$, abolishment of which could have significant effects on the global pattern of integrin heterodimers on the cell surface and therefore greatly alter the cell epithelial to mesenchymal transition, migration and adhesion [180].

In conclusion, our study provides important insights into the MEK-ERK1/2-dependent invasion of TGF β in type II endometrial cancer cells. In summary, we demonstrate that TGF β induced cell migration and adhesion are mediated by activated MEK-ERK1/2 signaling and its downstream effector integrin $\alpha v \beta 3$. Therapeutic approaches targeting the molecular mechanisms contributing to metastasis have the potential to significantly improve the clinical outcomes of patients with type II endometrial cancer.

5.4 Materials and methods

Cell culture

KLE and HEC-1B type II human endometrial cancer cell lines were purchased from the American Type Culture Collection (Manassas, VA). HEC-1B cells were cultured in Minimal Essential Medium (Gibco, Life Technologies, Burlington, ON) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories Inc., Logan, UT). KLE cells were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (Gibco, Life Technologies)

supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories Inc.). Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO₂ in air.

Antibodies and reagents

The following rabbit polyclonal antibodies were obtained from Cell Signaling Technology (Danvers, MA): human SMAD4 (#9515), human phospho-p44/42 MAPK (ERK1/2, Thr202/Tyr204; #9101), rat p44/42 MAPK (ERK1/2; #9102). The following rabbit monoclonal antibodies were obtained from Cell Signaling Technology: human phospho-SMAD2 (Ser465/467; 138D4), human phospho-SMAD3 (Ser423/425; C25A9), and human SMAD3 (C67H9). The mouse monoclonal antibodies used were: human SMAD2 (L16D3, Cell Signaling Technology), human integrin β 3 (#611141, BD Biosciences), human integrin α v (#611012, BD) and sea urchin α -tubulin (B-5-1-2, Santa Cruz). Horseradish peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were obtained from Bio-Rad Laboratories (Hercules, CA). SB431542 was purchased from Sigma-Aldrich (Oakville, ON). TGF β 1 was obtained from R&D Systems (Minneapolis, MN). The anti-integrin α v β 3 antibody, clone LM609, was purchased from Millipore (Darmstadt, Germany) and its control mouse IgG was obtained from Sigma (St. Louis, MO, USA).

Transwell migration assay

Cell culture inserts (24-well, pore size 8 μ m; BD Biosciences) were seeded with 1×10^5 cells in 250 μ L of medium supplemented with 0.1% FBS. Medium with 10% FBS (750 μ L) was added to the lower chamber and served as a chemotactic agent. After incubation for 24 h, non-migrating cells were removed from the upper side of the membrane, and the cells on the lower

side of the membrane were fixed with cold methanol and air dried. Cells were stained with Crystal Violet and counted using a light microscope. Each individual experiment was performed with triplicate inserts and five microscopic fields were counted per insert.

Adhesion assays

96-well plates were coated overnight at 4°C with vitronectin (1 µg/cm²; R&D Systems), fibronectin (10 µg/cm²; R&D Systems), or collagen IV (10 µg/cm²; R&D Systems) and then blocked for 1 h with 0.5% bovine serum albumin. Cells were seeded at a density of 4×10^4 cells/well and incubated at 37°C for 1.5 h. Non-adherent cells were removed by washing with PBS, and adherent cells were fixed with cold methanol and stained with 0.1% crystal violet for 25 minutes at room temperature. After removing the crystal violet solution, the stained cells were washed with water and 10% acetic acid was added to dissolve the crystal violet. Absorbances were measured at 590 nm using a microplate spectrophotometer.

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

Total RNA was extracted using TRIzol reagent (Invitrogen, Life Technologies, Burlington, ON) in accordance with the manufacturer's instructions. Reverse transcription was performed with 2 µg RNA, random primers and M-MLV reverse transcriptase (Promega, Madison, WI). Each 20 µl SYBR Green RT-qPCR reaction contained 1×SYBR Green PCR Master Mix (Applied Biosystems), 12 ng cDNA and 150 nM of each specific primer. The primers used were: integrin β₃, 5'-GAA GGC TGG CAG GCA TTG-3' (forward) and 5'-AAT GAT TGT CAC TAC CAA CAT GAC ACT-3' (reverse); integrin α_v, 5'-TGC CCA GCG CGT CTT C-3' (forward) and 5'-TGG GTG GTG TTT GCT TTG G-3' (reverse); SMAD4, 5'-TGG CCC AGG ATC AGT AGG

T-3' (forward) and 5'-CAT CAA CAC CAA TTC CAG CA-3' (reverse) and GAPDH, 5'-GAG TCA ACG GAT TTG GTC GT-3'(forward) and 5'- GAC AAG CTT CCC GTT CTC AG-3'(reverse). RT-qPCR was performed using an Applied Biosystems 7300 Real-Time PCR System equipped with 96-well optical reaction plates. The specificity of each assay was validated by melting curve analysis and agarose gel electrophoresis of the PCR products. Assay performance was validated by assessing amplification efficiencies by means of calibration curves, and ensuring that the plot of log input amount versus ΔC_q has a slope with an absolute value <0.1 . At least three separate experiments were performed and each sample was assayed in triplicate. A mean value of the triplicates was used for the determination of relative mRNA levels by the comparative C_q method with GAPDH as the reference gene and using the formula $2^{-\Delta\Delta C_q}$.

Western blot

Cells were lysed in ice cold lysis buffer (Cell Signaling Technology) with added protease inhibitor cocktail (Sigma-Aldrich). Extracts were centrifuged at $20,000\times g$ for 10 min at 4°C and supernatant protein concentrations were determined using the DC Protein Assay (Bio-Rad Laboratories). Equal amounts of protein (50 μg) were separated by SDS polyacrylamide gel electrophoresis and transferred onto PVDF membranes. After blocking for 1 h with 5% non-fat dry milk in TBS, the membranes were incubated overnight at 4°C with primary antibodies that were diluted 1000-fold in 5% non-fat milk-TBS. Following primary antibody incubation, the membranes were incubated with the appropriate HRP-conjugated secondary antibody. Immunoreactive bands were detected using enhanced chemiluminescent substrate or Super Signal West Femto chemiluminescent substrate (Thermo Fisher) and X-ray film. Membranes

were stripped with stripping buffer (50 mM Tris-HCl pH 7.6, 10 mM β -mercaptoethanol, and 1% SDS) at 50°C for 30 minutes and reprobbed with anti- α -tubulin antibody. Densitometric quantification was performed using Scion Image software (Scion Corp, Frederick, MD) with α -tubulin as the internal control for normalization.

Small interfering RNA (siRNA) transfection

To knock down endogenous integrin β 3, integrin α v and SMAD4, forty percent confluent cells were transfected for 48 hours with 20 nM ON-TARGETplus SMART pool siRNA targeting human integrin β 3, integrin α v and SMAD4 (Dharmacon, Lafayette, CO) using Lipofectamine RNAiMAX (Invitrogen, Life Technologies). ON-TARGETplus Non-targeting pool siRNA (Dharmacon) was used as the control.

Statistical analysis

Results are presented as the mean \pm SEM of at least three independent experiments. For experiments involving only two groups, results were analyzed by Two-Sample t-test assuming unequal variances using Excel. Multiple group comparisons were analyzed by one-way ANOVA followed by Student-Newman-Keuls test using PRISM software (GraphPad Software). Means were considered significantly different if $P < 0.05$ and are indicated by different letters.

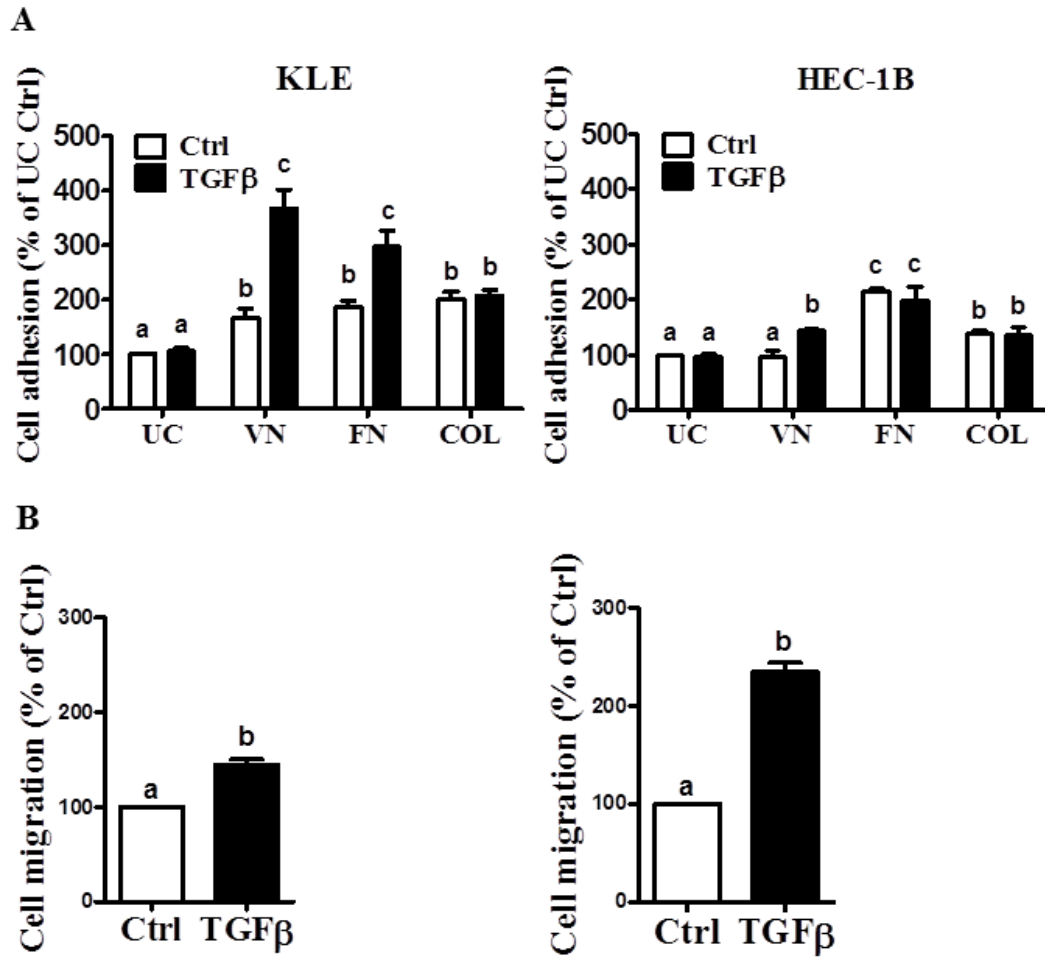
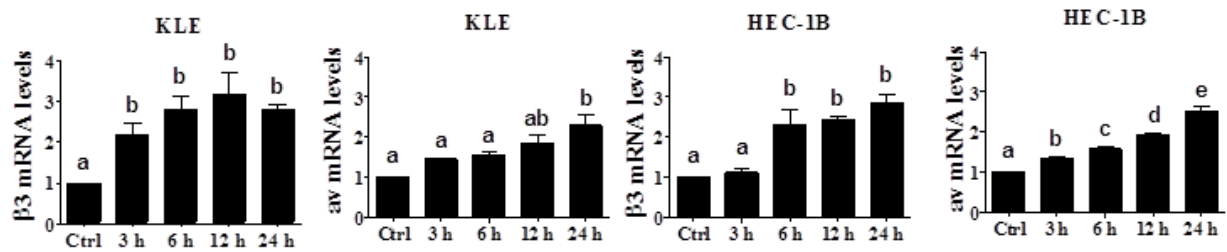


Figure 5. 1 TGFβ1 increases endometrial cancer cell adhesion and migration.

A, KLE and HEC-1B cells were treated with 10 ng/mL TGFβ1 for 24 h and then subjected to adhesion assay in un-coated (UC) plates or plates coated with vitronectin (VN), fibronectin (FN) or collagen IV (COL). B, KLE and HEC-1B cells were treated with 10 ng/mL TGFβ1 for 24 h and then seeded in transwell inserts for migration assay. Results are expressed as the mean ± SEM of at least three independent experiments. Values without a common letter are significantly different ($P < 0.05$).

A



B

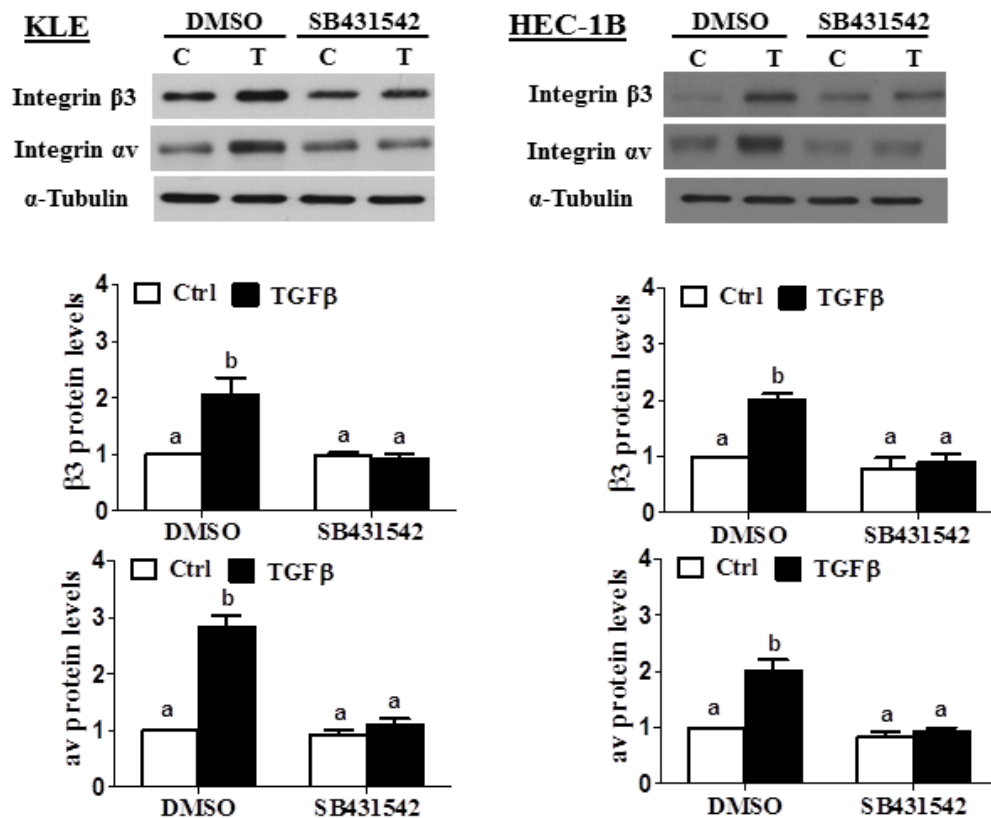


Figure 5. 2 TGF β 1 up-regulates integrin $\beta 3$ and αv expression in endometrial cancer cells.

A, KLE and HEC-1B cells were treated for varying times without (Ctrl; time-matched controls displayed as single bar) or with 10 ng/mL TGF β 1. Integrin $\beta 3$ and αv mRNA levels were measured by RT-qPCR. B, KLE and HEC-1B cells were pre-treated with vehicle control (DMSO) or SB431542 (10 μ M) for 1 h and then treated with 10 ng/mL TGF β 1 (T) for 24 h. Protein levels of integrin $\beta 3$ and αv were examined by Western blot (quantified data are normalized to α -

tubulin). Results are expressed as the mean \pm SEM of at least three independent experiments.

Values without a common letter are significantly different ($P < 0.05$).

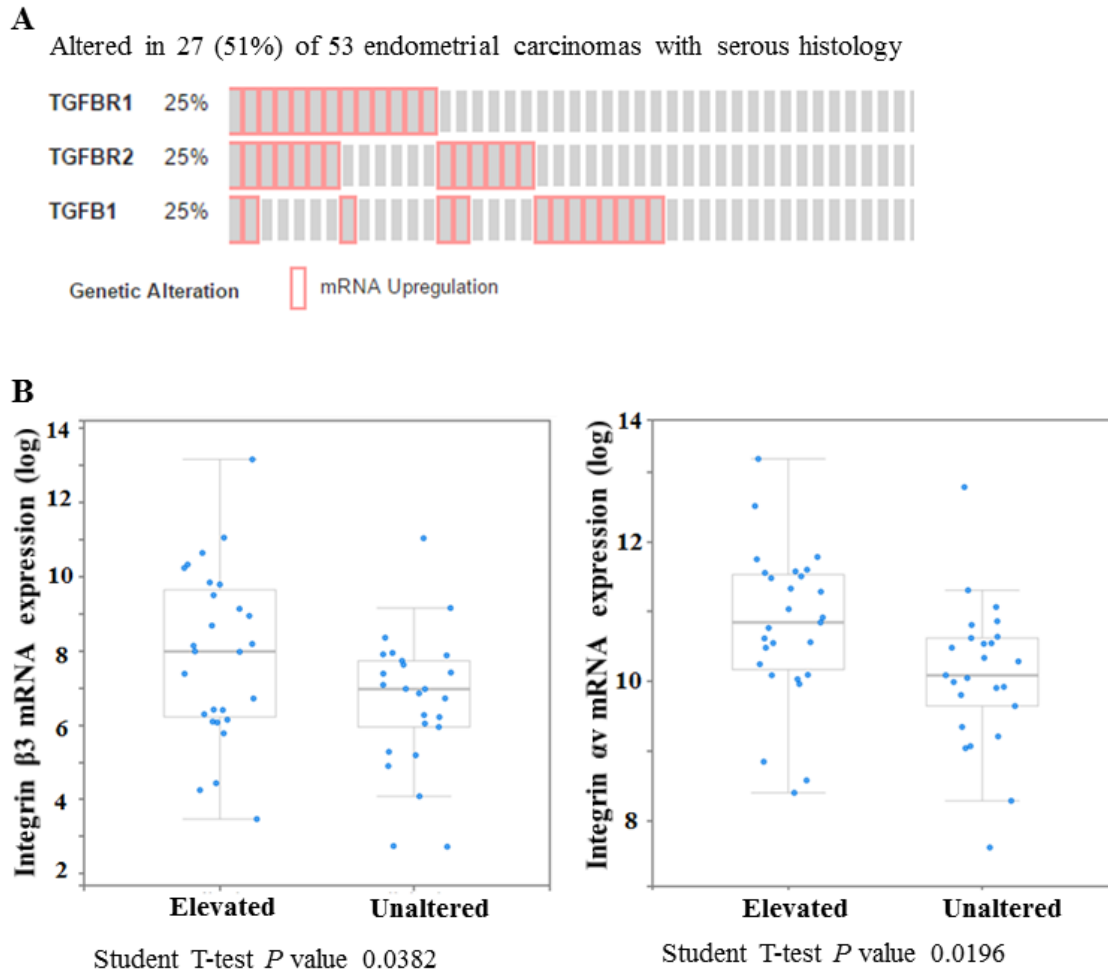


Figure 5. 3 Enhanced TGF β signaling may contribute to the up-regulation of integrin $\beta 3$ and αV in serous endometrial cancers.

The cBioPortal for Cancer Genomics was used to query serous endometrial carcinomas from The Cancer Genome Atlas (n=53) for up-regulation (upper quartile) of at least one component of the TGF β ligand-receptor gene set, including TGF $\beta 1$ (TGFB1), type I receptor TGFBR1, and type II receptor TGFBR2. (A) OncoPrint showing cases with elevated mRNA levels of TGFB1, TGFBR1 and TGFBR2 across all 53 serous endometrial carcinomas. (B) Enrichment analysis comparing integrin $\beta 3$ (*left*) and αV (*right*) mRNA levels between unaltered samples and those with TGFB1, TGFBR1 and TGFBR2 mRNA levels in the upper quartile (elevated). Expression

levels of integrin $\beta 3$ (left) and αv (right) mRNA (from RNA-Seq V2 RSEM) are displayed as boxplots with a P value from a Student T-test.

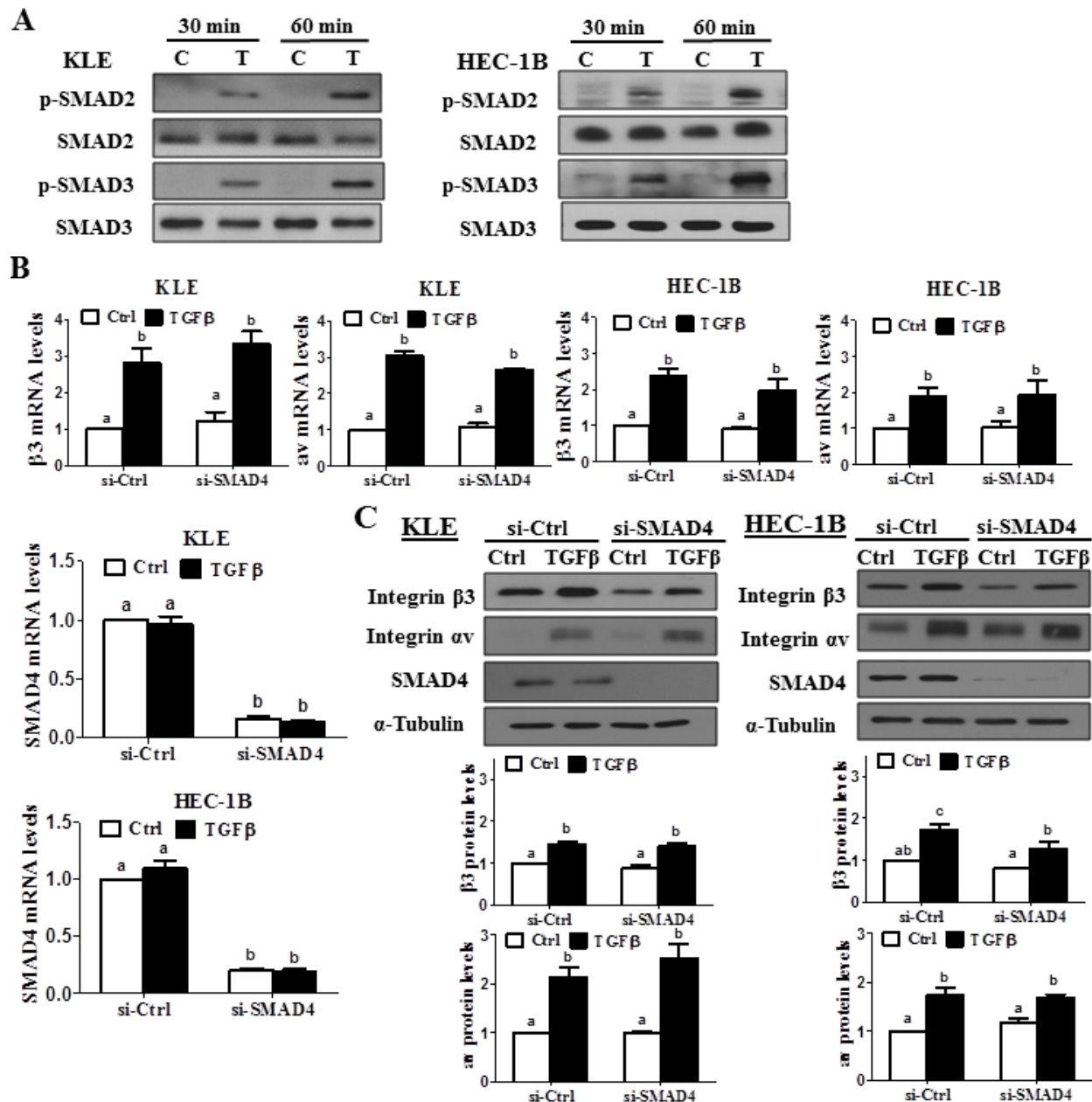


Figure 5. 4 SMAD2 and SMAD3 are not required for TGFβ1-induced up-regulation of integrin β3 and αv.

A, KLE and HEC-1B cells were treated without (C) or with 10 ng/mL TGFβ1 (T) for 30 or 60 min and Western blot was used to measure the levels of phosphorylated SMAD2 (p-SMAD2) and SMAD3 (p-SMAD3) in relation to their total levels (SMAD2 and SMAD3, respectively). B and C, KLE and HEC-1B cells were transfected for 48 h with 20 nM control siRNA (si-Ctrl) and

SMAD4 siRNA (si-SMAD4) and then treated without (Ctrl) or with 10 ng/mL TGF β 1 for 24 h. Integrin β 3, α v and SMAD4 mRNA and protein (C) levels were measured by RT-qPCR and Western blot, respectively. Results are expressed as the mean \pm SEM of at least three independent experiments and values without common letters are significantly different ($P < 0.05$).

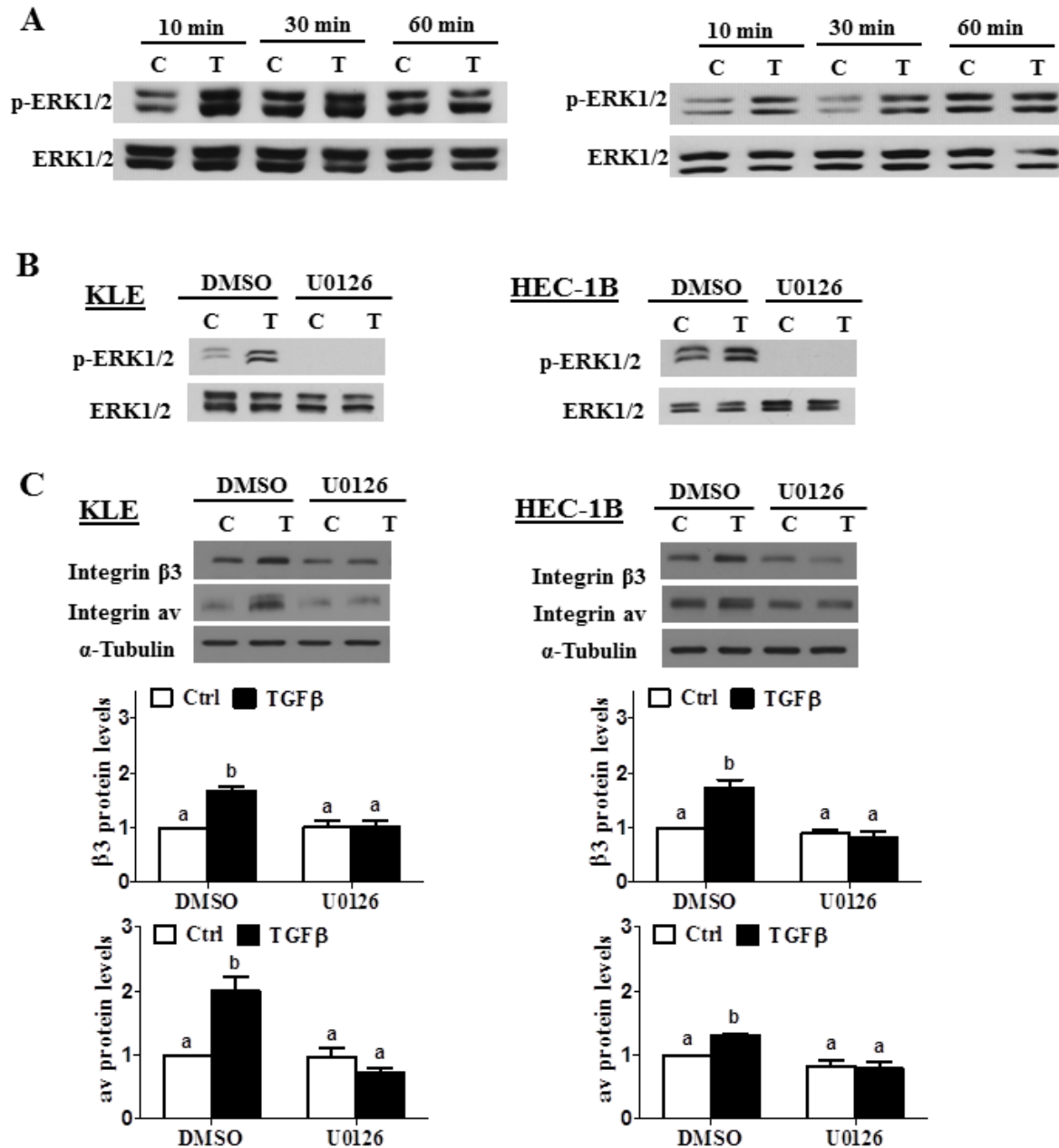


Figure 5. 5 MEK-ERK1/2 signaling is required for the up-regulation of integrin β3 and αv.

A, KLE and HEC-1B cells were treated without (C) or with 10 ng/mL TGFβ1 (T) for 10, 30 or 60 min and Western blot was used to examine the levels of phosphorylated ERK1/2 (p-ERK1/2) in relation to total levels of ERK1/2. B, KLE and HEC-1B cells were pre-treated with vehicle control (DMSO) or U0126 (10 μM) for 1 h and then treated without (C) or with 10 ng/mL TGFβ1 (T) for 10 min. Western blot was used to measure ERK1/2 phosphorylation. C, KLE and

HEC-1B cells were pre-treated with vehicle control (DMSO) or U0126 (10 μ M) for 1 h and then treated without (C) or with 10 ng/mL TGF β 1 (T) for 24 h. Protein levels of integrin β 3 and α v were examined by Western blot. Results are expressed as the mean \pm SEM of at least three independent experiments and values without common letters are significantly different ($P < 0.05$).

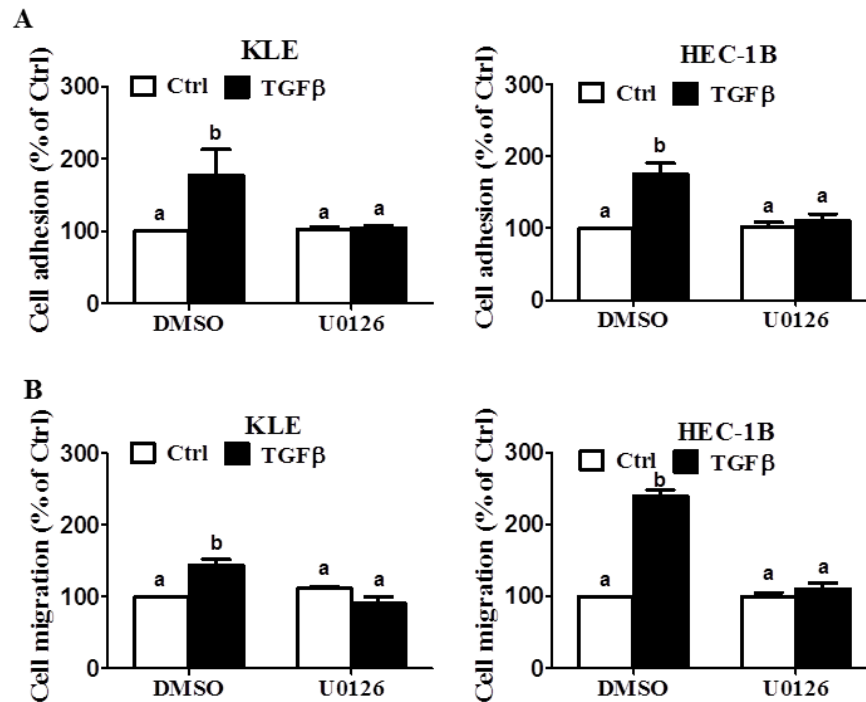


Figure 5. 6 MEK-ERK1/2 signaling is required for TGFβ1-induced cell adhesion to vitronectin and migration.

A and B, Vitronectin adhesion assays (A) and migration assays (B) were performed with KLE and HEC-1B cells following pre-treatment with vehicle control (DMSO) or U0126 (10 μM) for 1 h prior to treatment with or without 10 ng/mL TGFβ1 for 24 h. Results are expressed as the mean ± SEM of at least three independent experiments and values without common letters are significantly different ($P < 0.05$).

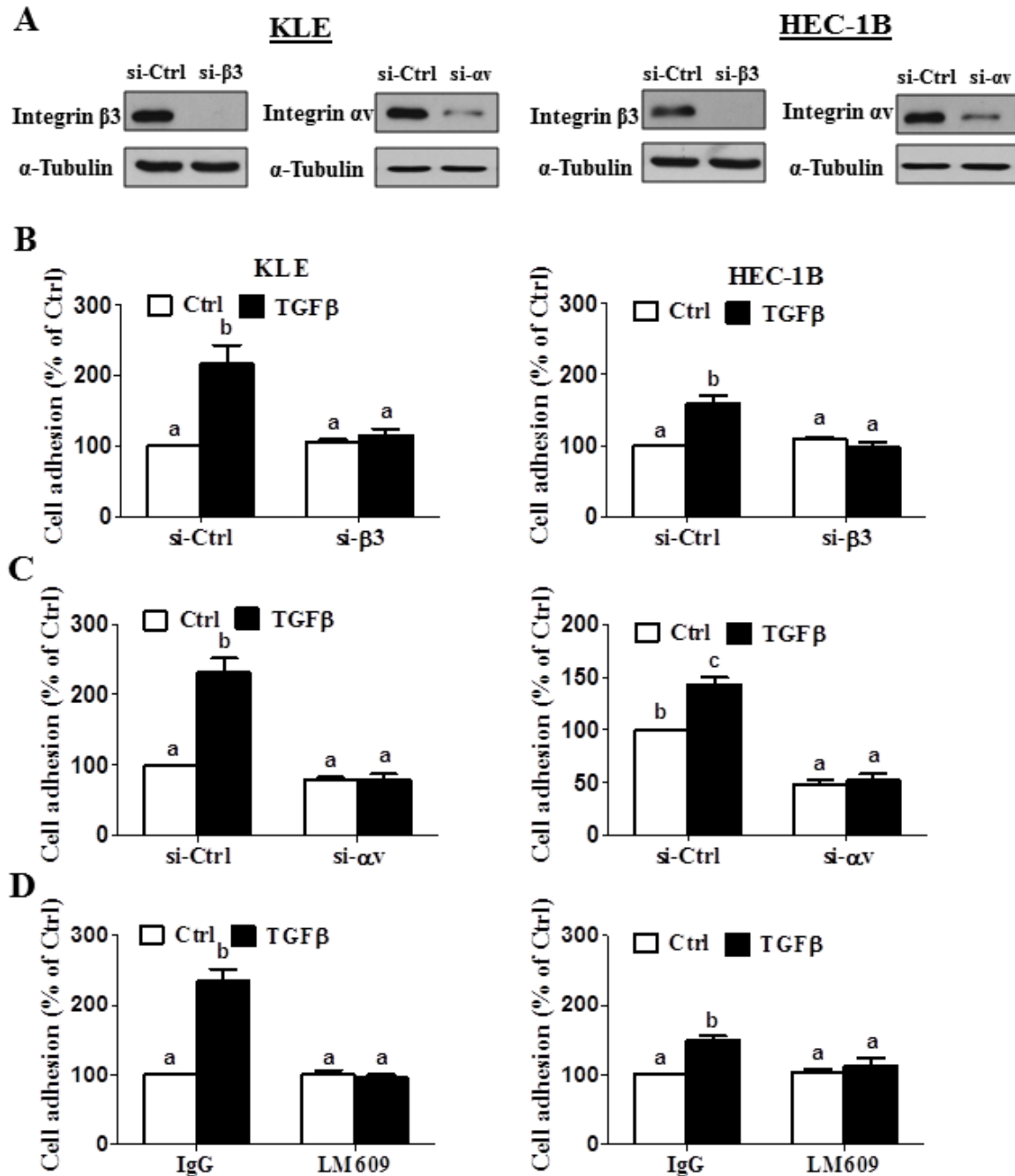


Figure 5. 7 Integrin β 3 and α v mediate TGF β 1-induced endometrial cancer cell adhesion to vitronectin.

A, KLE and HEC-1B cells were transfected for 48 h with 20 nM control siRNA (si-Ctrl) or integrin β 3 siRNA (si- β 3) or integrin α v (si- α v) and knockdown efficiencies were examined by Western blot. B and C, Vitronectin adhesion assays were performed with KLE and HEC-1B

following transfection with 20 nM control or integrin $\beta 3$ (B) or αv (C) siRNA for 48 h prior to treatment without (Ctrl) or with 10 ng/mL TGF β 1 for a further 24 h. D, Vitronectin adhesion assays were performed with KLE and HEC-1B following pre-treatment with control (IgG) or LM609 (10 μ g/ml) for 1 h prior to treatment with or without 10 ng/mL TGF β for 24 h. Results are expressed as the mean \pm SEM of at least three independent experiments. Values without a common letter are significantly different ($P < 0.05$).

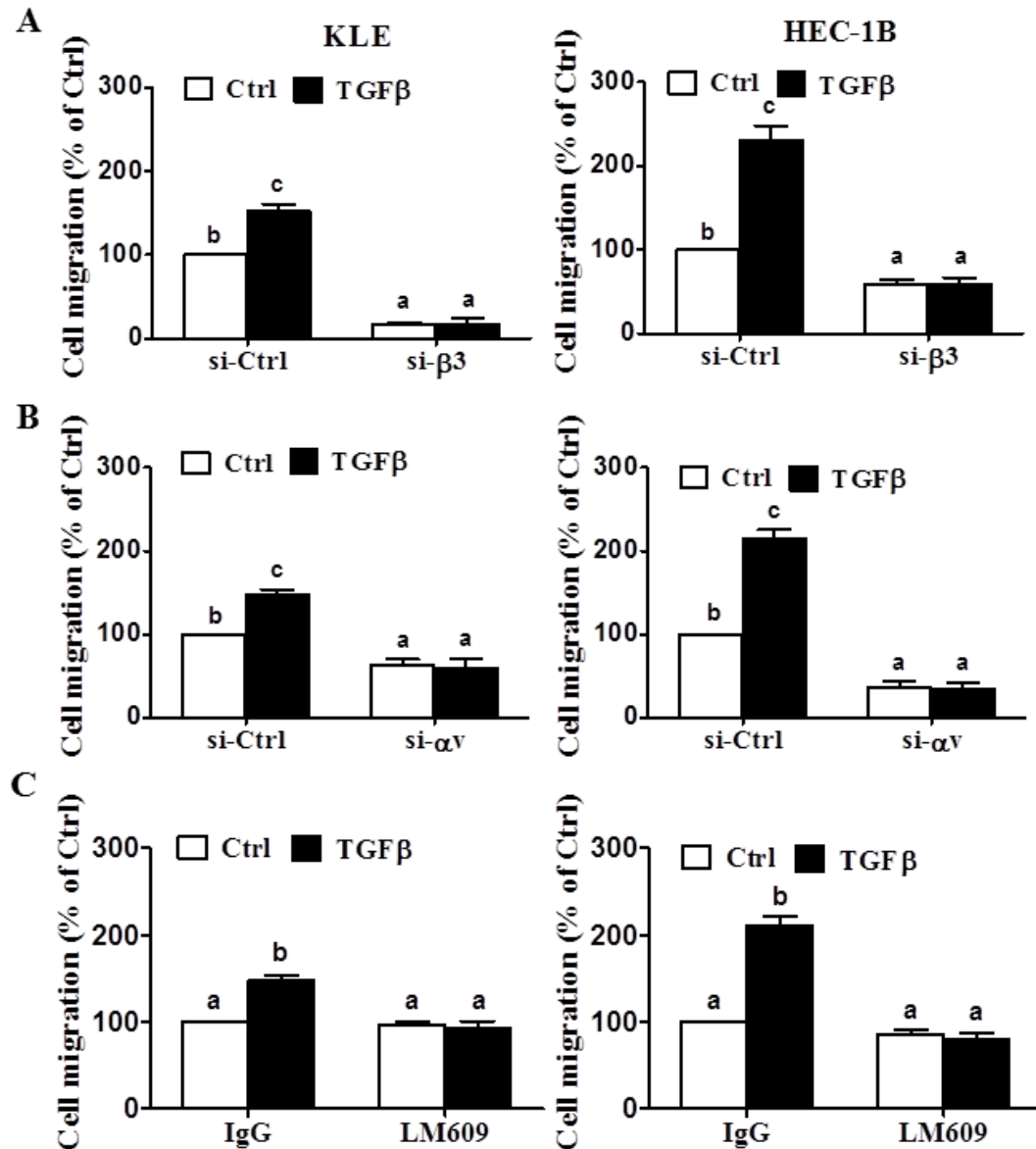


Figure 5. 8 Integrin $\beta 3$ and αv mediate TGF β -induced endometrial cancer cell migration.

A and B, Migration assays were performed with KLE and HEC-1B following transfection with 20 nM control or integrin $\beta 3$ (A) or αv (B) siRNA for 48 h prior to treatment without (Ctrl) or with 10 ng/mL TGF $\beta 1$ for a further 24 h. C, Migration assays were performed with KLE and HEC-1B following pre-treatment with control (IgG) or LM609 (10 μ g/mL) for 1 h prior to treatment with or without 10 ng/mL TGF β for 24 h. Results are expressed as the mean \pm SEM of

at least three independent experiments. Values without a common letter are significantly different ($P < 0.05$).

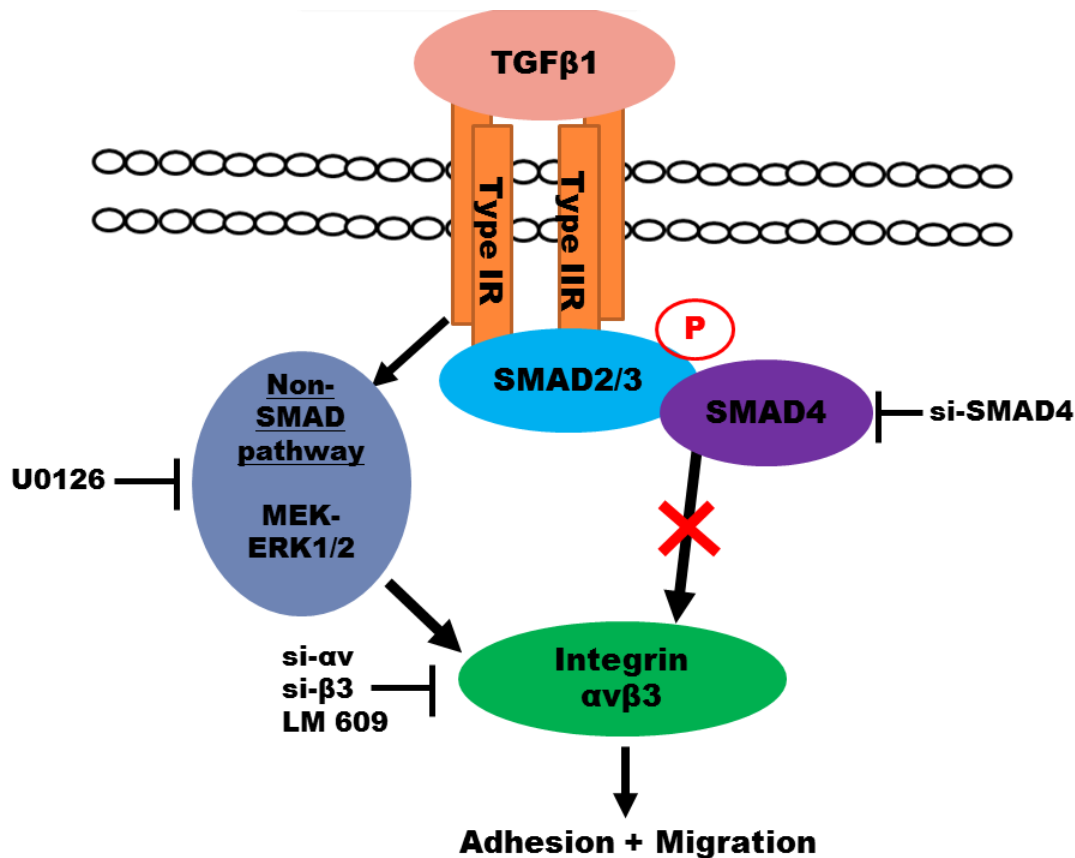


Figure 5. 9 A schematic illustration of Chapter 5 data

Treatment of KLE and HEC-1B cells with TGFβ1 generated comparable increases in cell adhesion and migration, integrin αvβ3 expression, ERK and SMAD2/3 phosphorylation (Figure 5.1, 5.2, 5.4 and 5.5). Knockdown of SMAD4 (Figure 5.4) failed to attenuate the effects of TGFβ1 on integrin αvβ3. Co-treatment with the MER-ERK1/2 inhibitor U0126 abolished TGFβ1 induced up-regulation of integrin αv, integrin β3, cell adhesion and migration (Figure 5.5 and 5.6). Importantly, TGFβ1 induced cell adhesion and migration were attenuated by siRNA mediated knockdown of integrin αvβ3 and LM609 (Figure 5.7).

Chapter 6: TGFβ1 stimulates type II endometrial cancer cell migration by down-regulating PTEN through SMAD and ERK1/2 signaling pathways

6.1 Introduction

Endometrial cancer is the second most lethal gynecological cancer in North America [8]. Type II endometrial cancer is a highly malignant subtype that accounts for the majority of endometrial cancer-related deaths, which is attributed to their propensity for extra-uterine spread [83, 197, 198]. Indeed, up to 90% of deaths associated with solid tumors are caused by chemotherapy-resistant metastases. Investigation into the molecular mechanisms behind type II endometrial cancer metastasis would thus provide insight for the development of improved therapies.

TGFβ1 belongs to the TGFβ super family, which regulates various biological functions, including cell proliferation, differentiation, migration and apoptosis [199]. Overexpression of TGFβ1 has been reported in various human cancers and correlates with metastasis and reduced survival rates. Furthermore, its immunoreactivity is stronger in invasive lymph node metastases than primary tumor sites [200]. TGFβ1 thus serves as a biomarker for poor prognosis and a potential therapeutic target in such malignancies [201, 202]. TGFβ1 mRNA, protein and receptors are expressed in normal and neoplastic human endometrial tissues or endometrial cancer cell lines [37, 173, 203, 204]. Moreover, immunohistochemical studies demonstrate that the expression of TGFβ1 is increased in the epithelial component of endometrial carcinomas compared with non-neoplastic tissues [37, 203], and the plasma level of TGFβ1 is greater in Stage-Ib and Stage-Ic patients than in Stage-Ia patients [38]. These results suggest that TGFβ1 may play a pivotal role in endometrial carcinogenesis in an autocrine and/or paracrine manner. Although early studies have demonstrated either inhibitory or no effects of TGFβ1 on the growth

of endometrial cancer cell lines [204-206], bioinformatic analyses of genetic and molecular relationships among genes altered in high-risk recurrent endometrial tumors demonstrate a critical role of TGF β 1 in the acquisition of a metastatic phenotype [41]. Indeed, TGF β 1 is found to enhance the invasiveness of HEC-1A and RL95-2 human endometrial cancer cells [41, 182]. These findings suggest that TGF β 1 exerts its tumor-promoting roles in human endometrial cancer mainly through the support of metastasis.

PTEN (phosphatase and tensin homologue) is a well-known tumor suppressor that antagonizes the PI3K/AKT pathway and is frequently mutated in human cancers. Loss of PTEN function causes hyperactivation of the PI3K/AKT pathway, altering cell growth, apoptosis, invasion and metastasis [78]. PTEN genetic mutations in endometrial cancer have been investigated and are reported to occur in 50-80% of type I endometrial cancer and in only 10% of type II cancers [7, 207]. Interestingly, the rate of PTEN protein loss in type II endometrial cancer is much higher than alterations in the PTEN gene [98, 99]. These findings indicate that loss of PTEN expression can be achieved by transcriptional or post-translational modulations. Indeed, it has been shown that treatment with TGF β 1 down-regulates PTEN protein levels by increasing its degradation in a human type II endometrial cancer cell line, KLE [100]. However, the degree to which PTEN expression can be transcriptionally regulated by TGF β 1 in human type II endometrial cancer cells and the mechanisms underlying this potential mode of regulation remain unclear.

In the present study, we show that TGF β 1 stimulates cell migration in two human type II endometrial cancer cell lines, KLE and HEC-50. Additionally, we show that both mRNA and protein levels of PTEN are down-regulated by TGF β 1 treatment. Overexpression of PTEN and

inhibition of the PI3K/AKT pathway abolished TGF β 1-stimulated cell migration. Moreover, using siRNA knockdown and a pharmacological inhibitor, we demonstrate that the SMAD and ERK1/2 signaling pathways are involved in TGF β 1-induced down-regulation of PTEN. Our findings indicate that PTEN may act as an important mediator in TGF β 1-stimulated type II endometrial cancer cell migration.

6.2 Results

TGF β 1 increases type II endometrial cancer cell migration

The survival rate of endometrial cancer drops from 90% to less than 17% once invasion and metastasis occur [8, 72]. Therefore, we first investigated the effect of TGF β 1 on the cell migration in two type II endometrial cancer cell lines, KLE and HEC-50. Boyden chamber transwell migration assay showed that treatment with 10 ng/mL TGF β 1 significantly increased the cell migration in both cell lines (Figure 6.1A). Moreover, the stimulatory effects of TGF β 1 on cell migration were abolished by pretreatment with a potent and specific TGF β type I receptor inhibitor, SB431542 (Figure 6.1B).

TGF β 1 down-regulates PTEN expression in type II endometrial cancer cells

It has been shown that KLE and HEC-50 cell lines express wild type of PTEN [208]. To examine the effect of TGF β 1 on PTEN expression, KLE and HEC-50 cells were treated with 10 ng/mL TGF β 1 for different periods of time. As shown in Figure 6.2A, treatment with TGF β 1 for 3 h significantly down-regulated PTEN mRNA levels in KLE cells and this effect was still observed after 24 h of treatment. The effect of TGF β 1 on PTEN mRNA down-regulation was also observed in HEC-50 cells, although the significance was detected after 6 h of TGF β 1

treatment. Western blot results further confirm the inhibitory effect of TGF β 1 on PTEN protein levels in both KLE and HEC-50 cells at 24 h. In addition, TGF β 1-induced down-regulation of PTEN protein levels were abolished by pretreatment with SB431542 (Figure 6.2B).

Overexpression of PTEN abolishes TGF β 1-stimulated cell migration

To examine whether down-regulation of PTEN contributes to the TGF β 1-stimulated cell migration, KLE and HEC-50 cells were transfected with vector encoded human PTEN-GFP. Western blot results showed that cells transfected with PTEN-GFP vector expressed PTEN-GFP protein while cells transfected with empty vector (pcDNA-GFP) did not (Figure 6.3A). The migration assay showed that basal levels of cell migration were decreased in PTEN overexpressing cells. Interestingly, TGF β 1-stimulated cell migration was abolished in the transfected cells overexpressing PTEN (Figure 6.3B). Taken together, these results indicated that PTEN played an important regulatory role in in type II endometrial cancer cell migration.

Inhibition of AKT activation attenuates TGF β 1-stimulated cell migration

Given the important role of PTEN in regulation of PI3K/AKT signaling pathway, we next examined whether overexpression of PTEN affects AKT activation. As shown in Figure 6.4A, in KLE and HEC-50 cells, treatment with TGF β 1 activated AKT signaling and this stimulatory effect was attenuated by overexpression of PTEN. Cells treated with 10% fetal bovine serum (FBS) served as a positive control. The migration assay results showed that pretreatments with two PI3K inhibitors, LY294002 and Wortmannin, attenuated TGF β 1-stimulated KLE and HEC-50 cell migration (Figure 6.4B).

Activation of SMAD signaling is involved in TGF β 1-induced down-regulation of PTEN expression

To the best of our knowledge, thus far, the underlying molecular mechanisms that mediate TGF β 1-induced down-regulation of PTEN in type II endometrial cancer cells remain unclear. TGF β 1 treatment significantly activated SMAD2 and SMAD3 in KLE and HEC-50 cells (Figure 6.5A). To directly examine the involvement of SMAD signaling pathways in TGF β 1-induced down-regulation of PTEN expression, siRNA-mediated knockdown of common SMAD, SMAD4, approach was used to block SMAD signaling pathways. As shown in Figure 6.5B, knockdown of SMAD4 attenuated TGF β 1-induced down-regulation of PTEN mRNA levels in both KLE and HEC-50 cells. Western blot results showed the similar effects that knockdown of SMAD4 attenuated the TGF β 1-induced down-regulation of PTEN protein levels (Figure 6.5C).

Activation of ERK1/2 signaling is involved in TGF β 1-induced down-regulation of PTEN expression

In the regulation of tumorigenesis, ERK1/2 signaling is closely interrelated with TGF β 1 system [176]. In addition, activations of ERK1/2 and TGF β 1 signaling pathways are associated with the high recurrence risk of endometrial cancer [41]. Therefore, we tested whether ERK1/2 signaling is also involved in TGF β 1-induced down-regulation of PTEN expression. As shown in Figure 6.6A, TGF β 1 treatment activated ERK1/2 signaling in both KLE and HEC-50 cells. The activations of ERK1/2 induced by TGF β 1 treatment were abolished by pretreatment with SB431542. In addition, pretreatment with specific MEK inhibitor, U0126, not only decreased basal activation of ERK1/2 but also abolished TGF β 1-induced activation of ERK1/2. Moreover, inhibition of ERK1/2 signaling abolished the suppressive effect of TGF β 1 on PTEN protein

levels (Figure 6.6B). These results indicated that ERK1/2 signaling was involved in TGF β 1-induced down-regulation of PTEN expression in type II endometrial cancer cells. Moreover, inhibition of SMAD and ERK1/2 pathways simultaneously exhibited similar attenuation in TGF β 1 induced decreases in PTEN protein expression (Figure 6.6C).

6.3 Discussion

Endometrial cancers are classified into two general clinicopathological types [102]. Type I endometrial cancers, which account for ~70% of endometrial cancers, are primarily composed of low-grade endometrioid tumors and are associated with a favorable prognosis. Type II cancers are predominantly non-endometrioid (serous and clear cell) tumors and are often of an advanced stage and correspond with poor survival [7, 86]. It has been shown that in type I cancers, the levels of SMAD2 phosphorylation are weak or undetectable, and the expression levels of type I and type II TGF β receptors are decreased. Moreover, primary cultures of type I cancers do not respond to TGF β 1-mediated growth inhibition [173]. These results suggest that TGF β 1 signaling is abrogated at an early stage of tumorigenesis of type I cancers, leading to their escape from normal modes of growth control. However, to date, the roles of TGF β 1 in the regulation of tumor progression in type II cancers remain unclear. Our results showed that SMAD2 and SMAD3 were expressed in two human type II endometrial cancer cell lines, KLE and HEC-50. In addition, both SMAD2 and SMAD3 were activated by TGF β 1 treatment. Importantly, inhibition of SMAD signaling pathways by knockdown of SMAD4 attenuated TGF β 1-induced down-regulation of PTEN expression. These results suggest that in the later stages of tumor progression, endometrial cancer cells can derive a selective advantage from the core components of the TGF β 1 pathway, advancing their growth and invasion to other tissues.

To date, various approaches targeting the TGF β 1 pathway are under preclinical or clinical investigation and have been shown to exhibit anti-tumor activity [209]. However, only a handful of studies emphasize the therapeutic potential of abrogating the TGF β 1 pathway in endometrial cancer. One study shows that ectopic expression of the dominant-negative TGF β 1 type II receptor in a mouse model of endometrial cancer cells significantly inhibits its metastasis to the lungs [182]. Mechanistically, many pro-oncogenic responses to TGF β 1 require the participation of SMAD-independent pathways [210], such as the ERK1/2 signaling pathway, which has a sophisticated and intimate relationship with the TGF β 1 system in regulating tumorigenesis [176]. Recent studies suggest that activation of ERK1/2 signaling may convert the growth inhibitory effects of TGF β 1 to a pro-oncogenic outcome and the cooperation between the TGF β 1 pathway and activated ERK1/2 signaling is essential for the invasive phenotype in different types of human cancer [176, 209, 211]. In the present study, ERK1/2 signaling is activated by TGF β 1 in type II endometrial cancer cells and mediates TGF β 1-induced down-regulation of tumor suppressor PTEN. This is consistent with the finding that ERK1/2 signaling and TGF β 1 are simultaneously identified as key factors of high-risk recurrence in a bioinformatic analysis of a sizable cohort of endometrial cancers [41]. Thus, inhibition of ERK1/2 signaling may amputate the tumor-promoting arm of TGF β 1 signaling while retaining its tumor suppressive effects in endometrial cancer. Since MEK-ERK1/2 may be involved in cross-talk with the SMAD pathway [212, 213], we knocked down SMAD4 by siRNA before treatment with U0126 and TGF β 1. Our results indicated that the inhibitory effect of MEK-ERK1/2 pathway on PTEN expression is independent of SMAD signaling in our cell models. In a phase II study for endometrial cancer, the MEK inhibitor Selumetinib has demonstrated only limited single-agent activity [171]. This could be attributed to the presence of other activated oncogenic signaling pathways, such as

PI3K/AKT, Wnt/ β -catenin and epidermal growth factor receptor/HER2 [172]. Therefore, simultaneous inhibition of multiple oncogenic pathways may generate better therapeutic results. Indeed, to test this hypothesis, a clinical trial for endometrial cancer is ongoing to evaluate the combined treatment of a MEK inhibitor Trametinib with an AKT inhibitor, GSK2141795 (ClinicalTrials.gov Identifier: NCT01935973).

Mutation of the PTEN gene is rare in type II endometrial cancers. However, PTEN protein loss is frequently detected in type II endometrial cancers [98, 99]. These results strongly indicate that loss of PTEN expression in type II endometrial cancers can be caused by transcriptional or post-translational modulations. In liver cancer cells, TGF β 1 down-regulates PTEN expression by accelerating the turnover rate of PTEN mRNA and increasing ubiquitin-proteasome-mediated PTEN protein degradation, without affecting its transcription [214]. In KLE cells, PTEN protein degradation has been shown to be increased by TGF β 1 [100]. The present study provides evidence that, in addition to its post-translational regulation, the expression of PTEN can be transcriptionally regulated by TGF β 1 in type II endometrial cancers.

In endometrial cancer, activation of AKT signaling is observed in approximately 60% of patients with recurrent/metastatic disease [215]. In addition, aberrant activation of PI3K/AKT signaling is associated with a more aggressive pathology and poorer prognosis, irrespective of endometrial cancer subtype [216]. Dysregulated PTEN and subsequent hyper-activation of the PI3K/AKT pathway has been implicated in cell motility [217, 218], and regulation of PTEN/PI3K/AKT activity has been implicated in tumorigenesis and anticancer therapy resistance [219]. In the present study, we showed that overexpression of PTEN attenuated TGF β 1-induced activation of

AKT and cell migration in type II endometrial cancer cells. In addition, we showed that inhibition of AKT activation attenuated TGF β 1-stimulated cell migration. Consistent with previous findings, our results further support that PTEN acts as a tumor suppressor by inhibiting AKT activation, which plays important roles in regulating the progression of type II endometrial cancer.

In summary, our results show that TGF β 1 stimulates the migration of type II endometrial cancer cells. In addition, TGF β 1 treatment down-regulates both mRNA and protein levels of PTEN. Overexpression of PTEN or inhibition of AKT signaling abolishes TGF β 1-stimulated cell migration. Moreover, TGF β 1-induced down-regulation of PTEN is mediated by activation of SMAD and ERK1/2 signaling pathways. Our study provides important insights into the molecular mechanisms that mediate TGF β 1-induced metastasis of type II endometrial cancers.

6.4 Materials and methods

Cell culture

KLE and HEC-50 type II human endometrial cancer cell lines were purchased from the American Type Culture Collection (Manassas, VA) [23]. Both cell lines were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (Gibco, Life Technologies) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories Inc.). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air.

Antibodies and reagents

The rabbit polyclonal antibodies used in this study were: SMAD4 (#9515, Cell Signaling Technology), phospho-ERK1/2 (Thr202/Tyr204; #9101, Cell Signaling Technology), ERK1/2 (#9102, Cell Signaling Technology), phospho-AKT (Ser473; #9271, Cell Signaling Technology), AKT (#9271, Cell Signaling Technology). The rabbit monoclonal antibodies used in this study were: PTEN (#9559, Cell Signaling Technology), phospho-SMAD2 (Ser465/467; 138D4, Cell Signaling Technology), phospho-SMAD3 (Ser423/425; C25A9, Cell Signaling Technology), and SMAD3 (C67H9, Cell Signaling Technology). The mouse monoclonal antibodies used were: SMAD2 (L16D3, Cell Signaling Technology) and α -tubulin (B-5-1-2, Santa Cruz). Horseradish peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were obtained from Bio-Rad Laboratories (Hercules, CA). SB431542 and U0126 were purchased from Sigma-Aldrich (Oakville, ON). TGF β 1 was obtained from R&D Systems (Minneapolis, MN).

Transwell migration assays

Migration assays were performed in Boyden chambers. Cell culture inserts (24-well, pore size 8 μ m; BD Biosciences, Mississauga, ON) were seeded with 1×10^5 cells in 250 μ L of medium with 0.1% FBS. Un-coated inserts were used for migration assay. Medium with 10% FBS (750 μ L) was added to the lower chamber and served as a chemotactic agent. After incubation for 24 h, non-migrating cells were wiped from the upper side of the membrane and cells on the lower side were fixed in cold methanol and air dried. Cell nuclei were stained with crystal violet and counted. Each individual experiment had triplicate inserts and five microscopic fields were counted per insert.

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

Total RNA was extracted using TRIzol reagent (Invitrogen, Life Technologies, Burlington, ON) in accordance with the manufacturer's instructions. Reverse transcription was performed with 2 µg RNA, random primers and M-MLV reverse transcriptase (Promega, Madison, WI). The primers used for SYBR Green RT-qPCR were: PTEN, 5'-CGA ACT GGT GTA ATG ATA TGT -3' (forward) and 5'-CAT GAA CTT GTC TTC CCG T -3' (reverse) and GAPDH, 5'-GAG TCA ACG GAT TTG GTC GT-3' (forward) and 5'- GAC AAG CTT CCC GTT CTC AG-3' (reverse). RT-qPCR was performed using an Applied Biosystems 7300 Real-Time PCR System equipped with 96-well optical reaction plates. The specificity of each assay was validated by melting curve analysis and agarose gel electrophoresis of the PCR products. Assay performance was validated by assessing amplification efficiencies by means of calibration curves, and ensuring that the plot of log input amount versus ΔC_q has a slope $<|0.1|$. At least three separate experiments were performed and each sample was assayed in triplicate. A mean value of the triplicates was used for the determination of relative mRNA levels by the comparative C_q method with GAPDH as the reference gene and using the formula $2^{-\Delta\Delta C_q}$.

Western blot

Cells were lysed in ice cold lysis buffer (Cell Signaling Technology) with added protease inhibitor cocktail (Sigma-Aldrich). Extracts were centrifuged at 20,000×g for 10 min at 4°C and supernatant protein concentrations were determined using the DC Protein Assay (Bio-Rad Laboratories). Equal amounts of protein were separated by SDS polyacrylamide gel electrophoresis and transferred onto PVDF membranes. After blocking for 1 h with 5% non-fat dry milk in Tris-buffered saline (TBS), the membranes were incubated overnight at 4°C with

primary antibodies that were diluted 1000-fold in 5% non-fat milk-TBS. Following primary antibody incubation, the membranes were incubated with the appropriate HRP-conjugated secondary antibody. Immunoreactive bands were detected using enhanced chemiluminescent substrate or SuperSignal West Femto chemiluminescent substrate (Thermo Fisher) and X-ray film. Membranes were stripped with stripping buffer (50 mM Tris-HCl pH 7.6, 10 mM β -mercaptoethanol, and 1% SDS) at 50°C for 30 minutes and reprobed with anti- α -tubulin antibody. Densitometric quantification was performed using Scion Image software (Scion Corp, Frederick, MD) with α -tubulin as the internal control for normalization.

Plasmid constructs and transfection

pcDNA-GFP and pcDNA-PTEN-GFP were generously provided by Dr. Alonzo H. Ross (University of Massachusetts Medical School, Worcester, MA, USA). Cells at 60% confluence were transfected for 48 h with the pcDNA-GFP or pcDNA-PTEN-GFP vector using Lipofectamine 3000 (Life Technologies) according to the manufacturer's instructions.

Small interfering RNA (siRNA) transfection

To knock down endogenous SMAD4, 60% confluent cells were transfected for 48 h with 20 nM ON-TARGETplus SMART pool siRNA targeting human SMAD4 (Dharmacon, Lafayette, CO) using Lipofectamine RNAiMAX (Invitrogen, Life Technologies). ON-TARGETplus Non-targeting pool siRNA (Dharmacon) was used as the control.

Statistical analysis

The results are presented as the mean \pm SEM of at least three independent experiments. For

experiments involving only two groups, the results were analysed by a two-sample *t*-test assuming unequal variances using Excel. Multiple group comparisons were analysed by one-way ANOVA followed by Student-Newman-Keuls tests using PRISM software (GraphPad Software). The means were considered significantly different if $p < 0.05$ and are indicated by different letters.

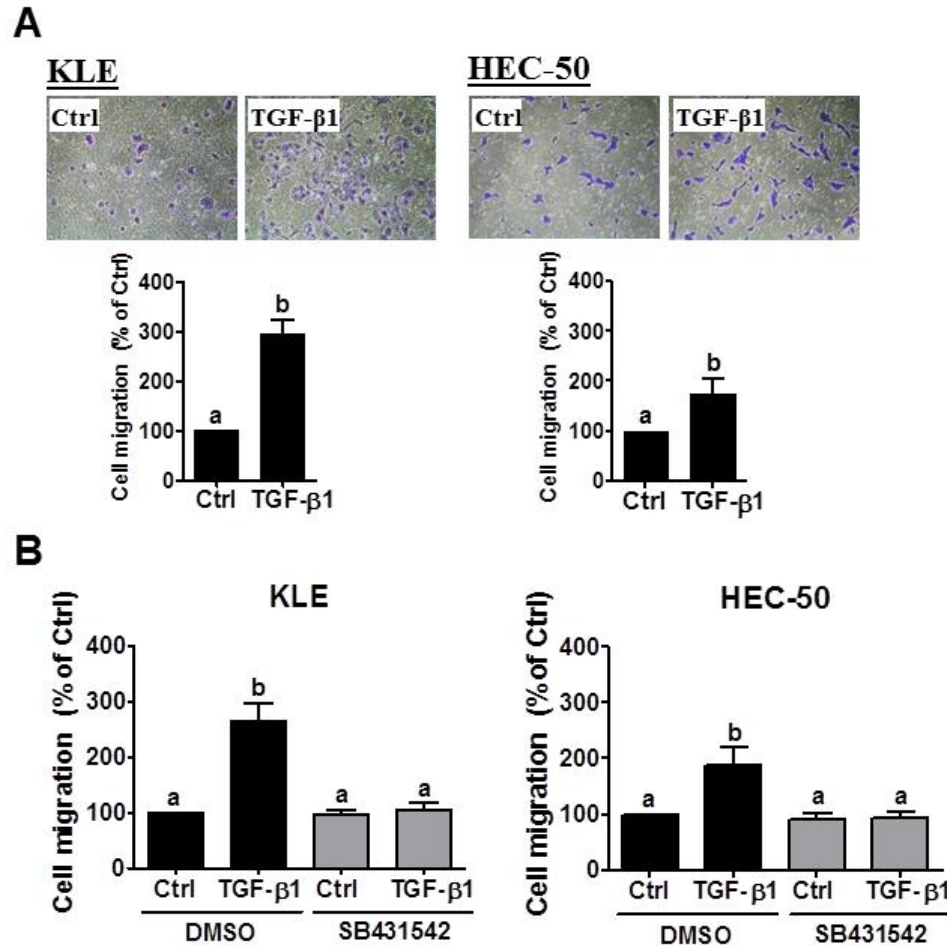


Figure 6. 1 TGFβ1 stimulates type II endometrial cancer cell migration.

A, KLE and HEC-50 cells were treated without (Ctrl) or with 10 ng/mL TGFβ1 for 24 h and then seeded into transwell inserts for migration assay. Upper panels show representative photomicrographs of migrating cells, while lower panels show summarized quantitative results.

B, KLE and HEC-50 cells were pretreated with vehicle (DMSO) or SB431542 (10 μM) for 1 h and then treated with 10 ng/mL TGFβ1 for 24 h. After treatment, the levels of cell migration were examined by migration assay. The results are expressed as the mean ± SEM of at least three independent experiments. Values without a common letter were significantly different ($p < 0.05$).

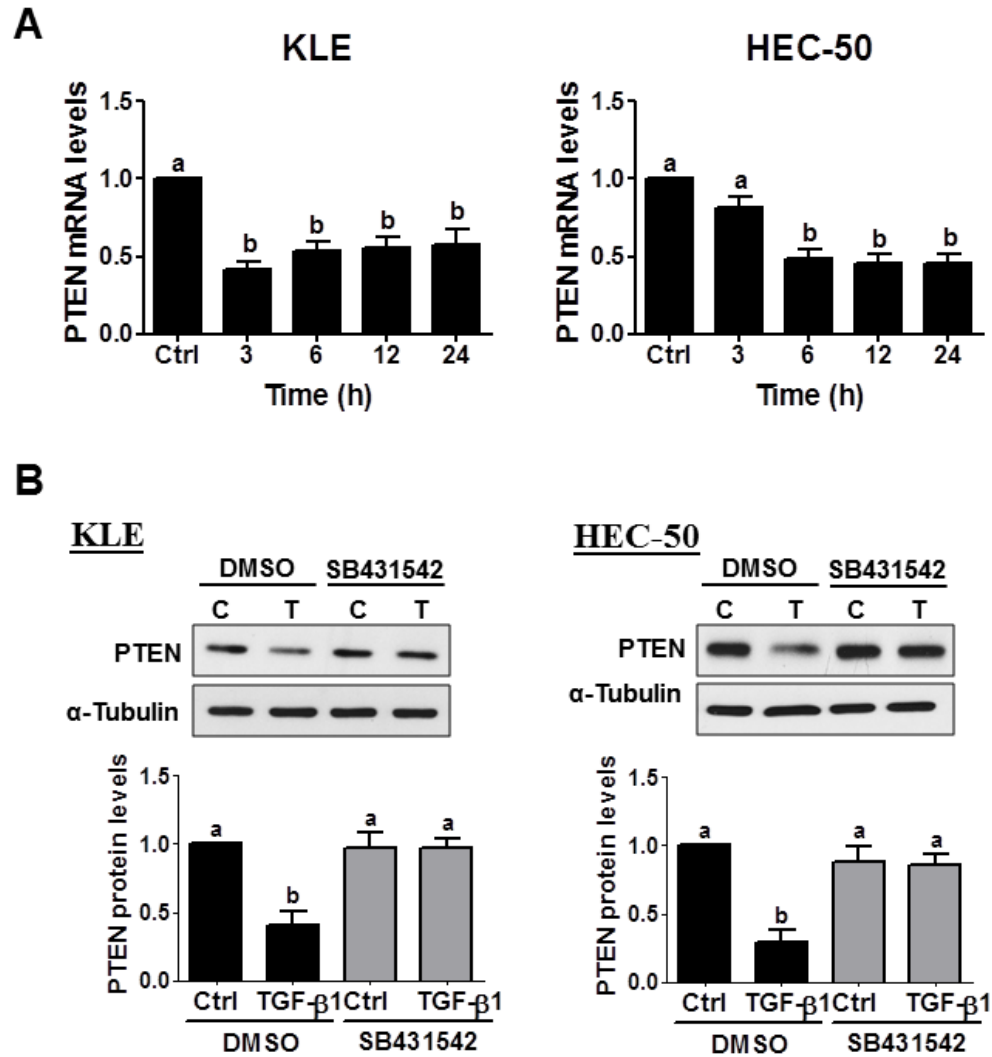


Figure 6. 2 TGF β 1 down-regulates PTEN expression in type II endometrial cancer cells.

A, KLE and HEC-50 cells were treated without (Ctrl) or with 10 ng/mL TGF β 1 for different periods of time, and the mRNA levels of PTEN were examined by RT-qPCR. The level of PTEN mRNA at each time point was normalized to the GAPDH mRNA level at the same time point. B, KLE and HEC-50 cells were pretreated with vehicle (DMSO) or SB431542 (10 μ M) for 1 h and then treated with 10 ng/mL TGF β 1 (T) for 24 h. The protein levels of PTEN were examined by Western blot. The results are expressed as the mean \pm SEM of at least three independent experiments. Values without a common letter were significantly different ($p < 0.05$).

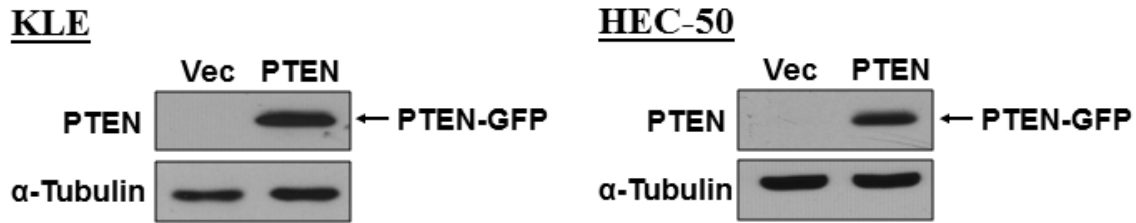
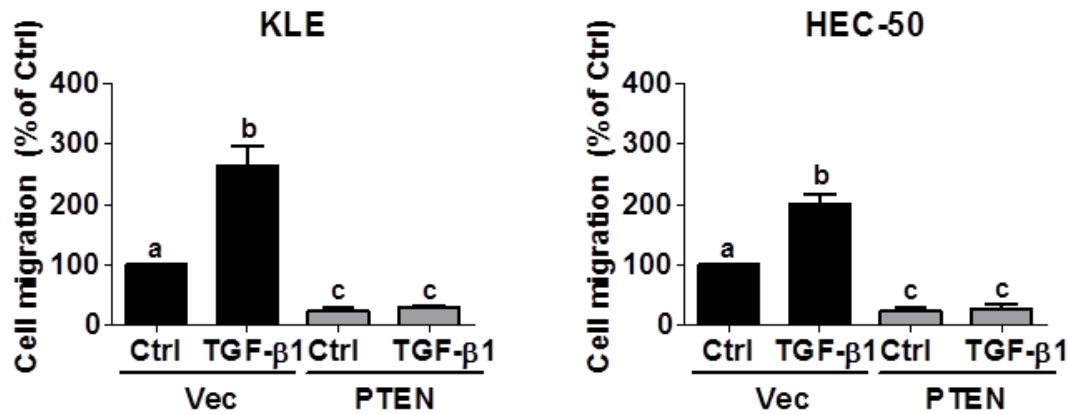
A**B**

Figure 6. 3 Overexpression of PTEN abolishes TGF β 1-stimulated cell migration.

A, KLE and HEC-50 cells were transfected with the 1 μ g pcDNA-GFP (Vec) or pcDNA-PTEN-GFP (PTEN) for 48 h. The protein levels of PTEN-GFP were examined by Western blot. B, KLE and HEC-50 cells were transfected with the 1 μ g pcDNA-GFP (Vec) or pcDNA-PTEN-GFP (PTEN) for 48 h and then treated with 10 ng/mL TGF β 1 for 24 h. After treatment, the levels of cell migration were examined by migration assay. The results are expressed as the mean \pm SEM of at least three independent experiments. Values without a common letter were significantly different (p < 0.05).

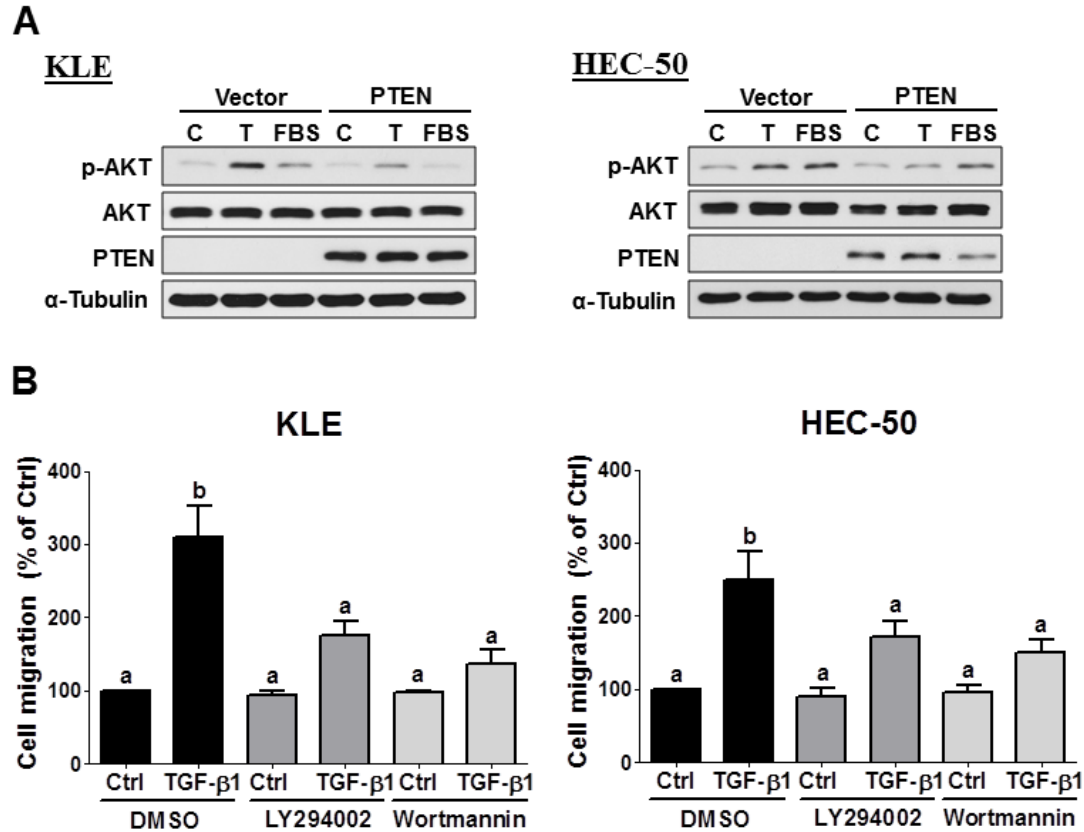


Figure 6. 4 Inhibition of AKT signaling attenuates TGF β 1-stimulated cell migration.

A, KLE and HEC-50 cells were transfected with the 1 μ g pcDNA-GFP (Vec) or pcDNA-PTEN-GFP (PTEN) for 48 h and then treated with 10 ng/mL TGF β 1 (T) or 10% fetal bovine serum (FBS) for 24 h. Phosphorylation of AKT was determined by Western blot using specific antibodies for phosphorylated (activated) form of AKT (p-AKT). The membrane was stripped and re-probed with antibody to the total AKT. The overexpression of PTEN-GFP protein was confirmed by Western blot. B, KLE and HEC-50 cells were pretreated with vehicle e (DMSO), LY294002 (10 μ M) or Wortmannin (1 μ M) for 1 h and then treated with 10 ng/mL TGF β 1 for 24 h. After treatment, the levels of cell migration were examined by migration assay. The results are expressed as the mean \pm SEM of at least three independent experiments. Values without a common letter were significantly different ($p < 0.05$).

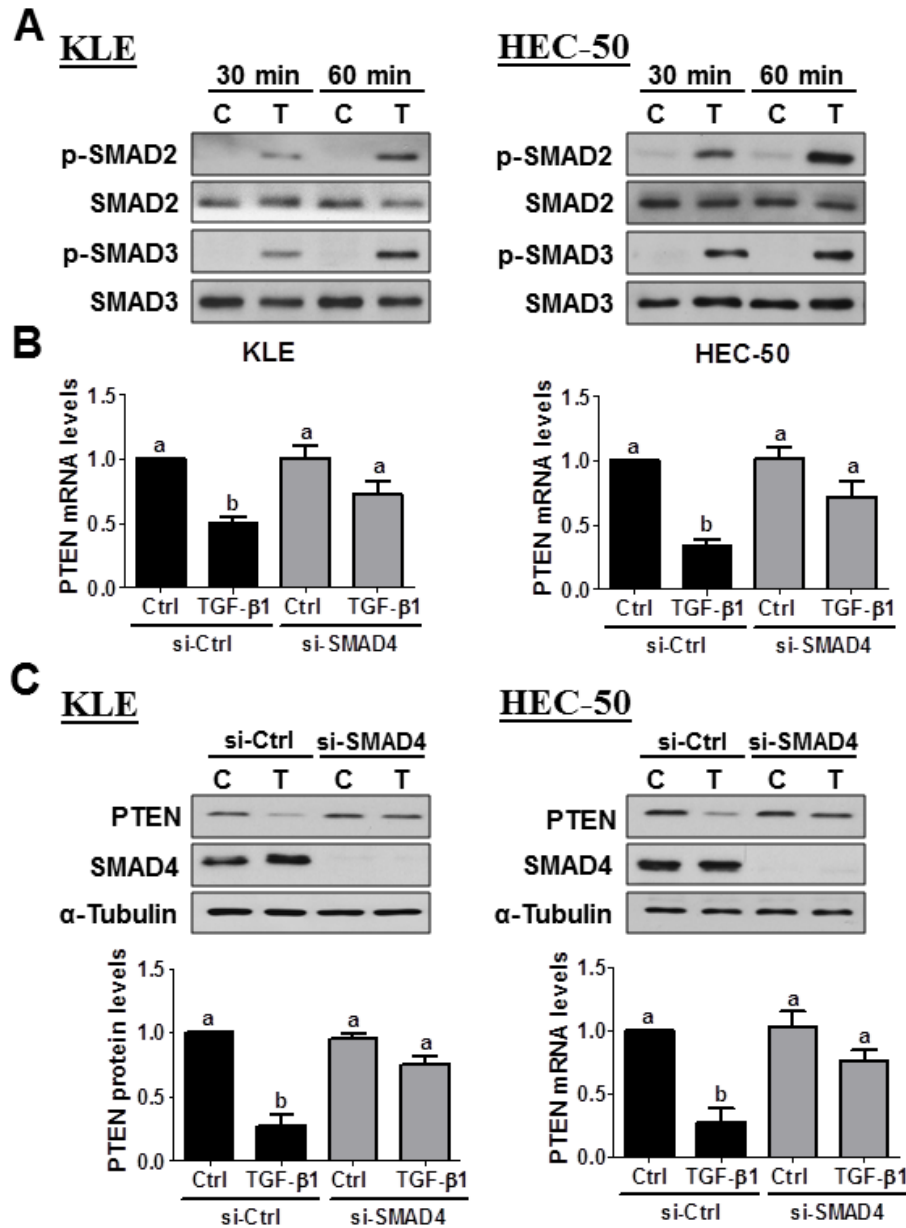


Figure 6. 5 Activation of SMAD signaling is required for TGFβ1-induced down-regulation of PTEN expression.

A, KLE and HEC-50 cells were treated with 10 ng/mL of TGFβ1 (T) for 30 and 60 minutes. Phosphorylation of SMAD2 and SMAD3 were determined by Western blot using specific antibodies for phosphorylated (activated) forms of SMAD2 (p-SMAD2) and SMAD3 (p-SMAD3). The membranes were stripped and re-probed with antibodies to the total SMAD2 and

SMAD3. B and C, KLE and HEC-50 cells were transfected with 20 nM of control siRNA (si-Ctrl) or SMAD4 siRNA (si-SMAD4) for 48 h and then treated with 10 ng/mL of TGF β 1 (T) for 24 h. The PTEN mRNA (B) and protein (C) levels were examined by RT-qPCR and Western blot, respectively. The results are expressed as the mean \pm SEM of at least three independent experiments. Values without a common letter were significantly different ($p < 0.05$).

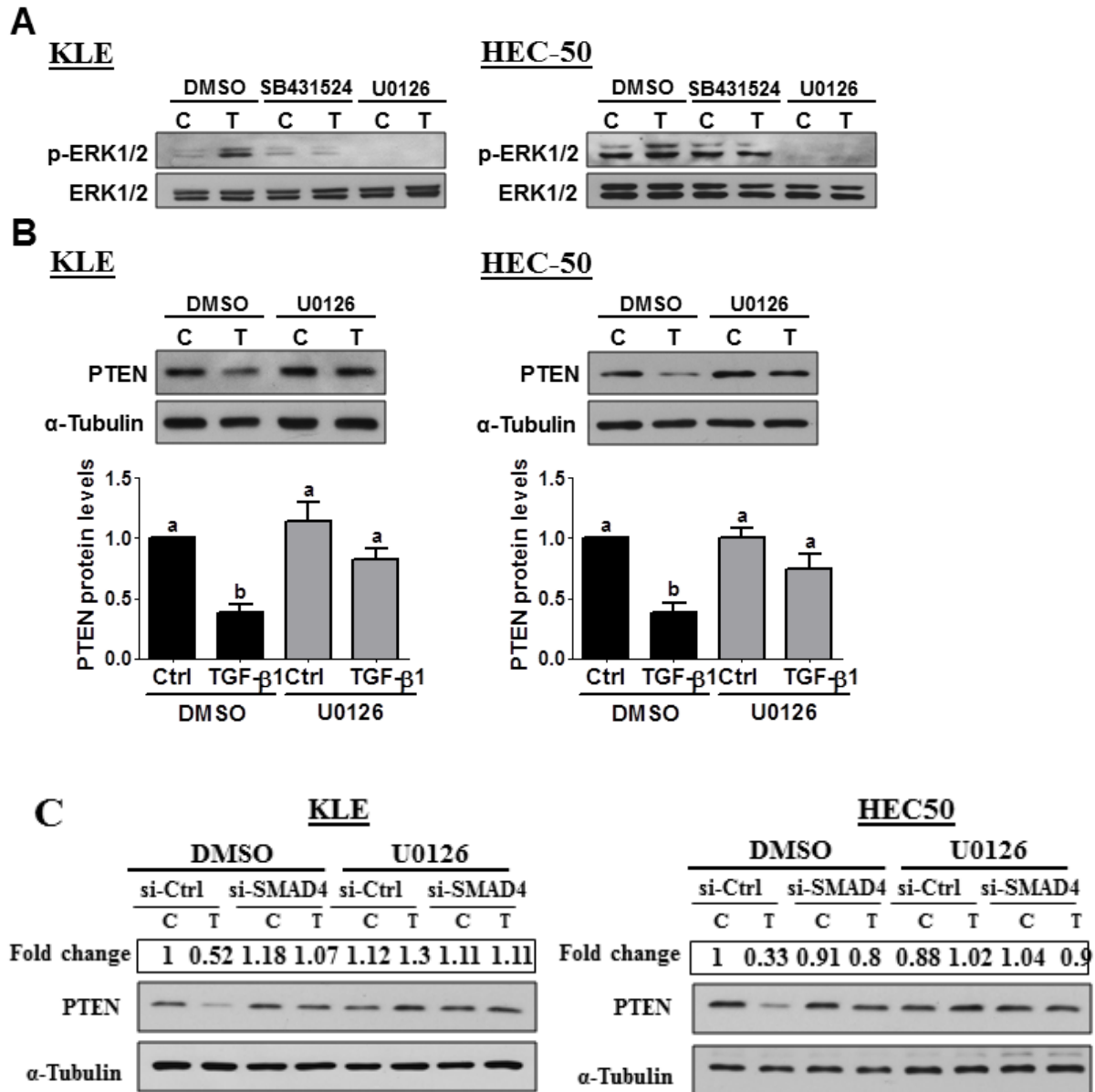


Figure 6. 6 Activation of ERK1/2 signaling is required for TGF β 1-induced down-regulation of PTEN expression.

A, KLE and HEC-50 cells were pretreated with vehicle (DMSO), SB431542 (10 μ M) or U0126 (10 μ M) for 1 h and then treated with 10 ng/mL TGF β 1 (T) for 10 min. Phosphorylation of ERK1/2 was determined by Western blot using specific antibody for phosphorylated (activated) form of ERK1/2. The membranes were stripped and re-probed with antibody to the total ERK1/2.

B, KLE and HEC-50 cells were pretreated with vehicle (DMSO) or U0126 (10 μ M) for 1 h and then treated with 10 ng/mL TGF β 1 (T) for 24 h. The PTEN protein levels were examined by Western blot. C, Cells were transfected with 50nM control siRNA (si-Ctrl) or Smad4 siRNA (si-Smad4) for 48 hours and then treated with 10 ng/mL TGF β 1 in combination with U0126 (10 μ M) for an additional 24 hours. PTEN protein levels were measured by Western blot. The results are expressed as the mean \pm SEM of at least three independent experiments. Values without a common letter were significantly different ($p < 0.05$).

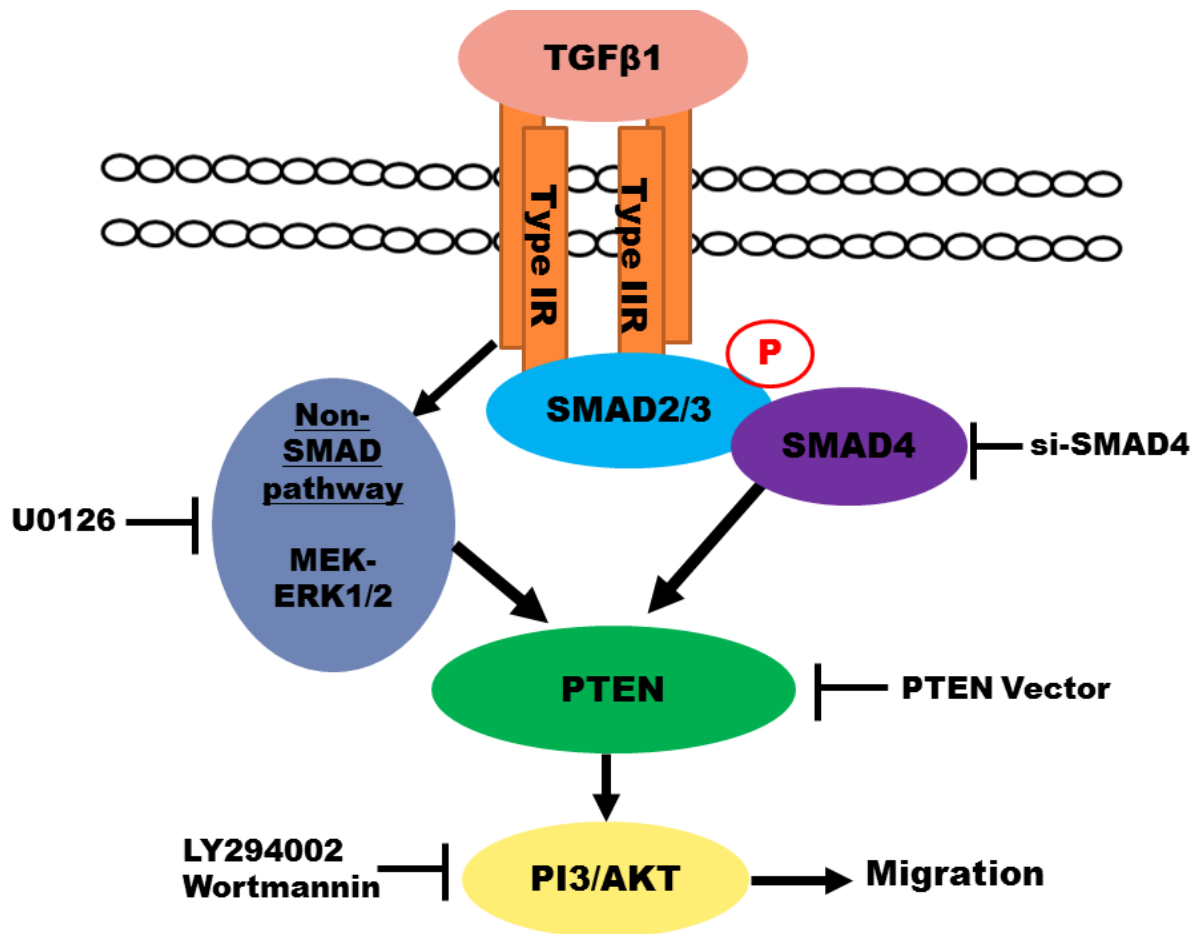


Figure 6. 7 A schematic illustration of Chapter 6 data.

Treatment of KLE and HEC-50 cells with TGFβ1 generated comparable increase in cell migration and decrease in PTEN expression (Figure 6.1 and 6.2), ERK and SMAD2/3 phosphorylation (Figure 6.5 and 6.6). Knockdown of SMAD4 (Figure 6.5) or co-treatment with the MEK-ERK1/2 inhibitor U0126 abolished TGFβ1 induced down-regulation of PTEN (Figure 6.6). Moreover, PTEN overexpression abolished TGFβ1 induced AKT phosphorylation and blocking AKT phosphorylation using AKT inhibitors attenuated TGFβ1 induced cell migration (Figure 6.4). Importantly, TGFβ1 induced cell migration was attenuated by vector mediated PTEN overexpression (Figure 6.3).

Chapter 7: Conclusion

7.1 Conclusion

The objective of the current thesis was to investigate the potential roles of TGF β subfamily: activin B and TGF β 1 in regulating human endometrial cancer cell adhesion and migration as well as the underlying molecular mechanisms. Our results provide insight into the cellular and molecular events regulated by activin B and TGF β 1 in endometrial cancer progression.

In Chapter 3, the effects of activin B on type II endometrial cancer cell adhesion to different ECM, migration and invasion, and the underlying molecular mechanisms, particularly the roles of integrin β 3 and SMAD2/3 signaling were studied. Activin B increased KLE and HEC-1B cell migration, invasion and adhesion to vitronectin. Moreover, activin B treatment increased integrin β 3 mRNA and protein levels via SMAD2/3-SMAD4 signaling. Importantly, siRNA knockdown studies revealed that integrin β 3 is required for basal and activin B-induced cell migration, invasion and adhesion. Thus, this study not only demonstrated the biological functions of activin B and potential mechanisms of poor survival, but also unraveled novel therapeutic targets such as activin B signaling and integrin β 3 for type II endometrial cancers.

In Chapter 4, we continued to investigate the molecular mechanisms underlying the migration promoting effects of activin B on type II endometrial cancer cells: KLE and HEC-50. Since cancer cell migration is also closely associated with EMT which is characterized by the down-regulation of E-cadherin, we investigated the relationship between activin B signaling and E-cadherin. Activin B treatment significantly decreased E-cadherin expression in a time-dependent manner in KLE and HEC-50 cell lines. Interestingly, these effects were not inhibited by siRNA-

mediated knockdown of SMAD2, SMAD3 or SMAD4. Rather, the suppressive effects of activin B on E-cadherin were mediated by MEK-ERK1/2 signaling, which was abolished by MEK-ERK1/2 inhibitor U0126. Activin B also increased the expression of SNAIL at mRNA and protein levels, which were also blocked by U0126 pretreatment. Furthermore, activin B-induced cell migration was inhibited by forced-expression of E-cadherin or pre-treatment with the activin/TGF β type I receptor inhibitor SB431542 or the MEK inhibitor U0126. This study identified a novel SMAD-independent pathway linking enhanced activin B signaling to reduced E-cadherin expression and increased migration in type II endometrial cancer, suggesting approaches that inhibit both SMAD-independent and -dependent signalings may have clinical utility for the treatment of this highly lethal subtype of endometrial cancer.

Since TGF β signaling and activin signaling share the same canonical SMAD2/3-SMAD4 signaling and non-canonical signaling, such as AKT/PI3K, MAPK/ERK and Wnt/ β -catenin, we wonder if TGF β could exert similar effects on endometrial cancer as activin B. Therefore, the effects of TGF β 1 on endometrial cancer cell adhesion, migration and underlying mechanisms were studied. TGF β 1 increased KLE and HEC-1B cell adhesion to vitronectin and migration. Moreover, TGF β 1 treatment increased integrin β 3 and α v mRNA and protein levels. TGF β 1 induced the phosphorylation of SMAD2 and SMAD3, but siRNA-mediated depletion of SMAD4 failed to alter the stimulatory effects of TGF β 1 on integrin β 3 or α v mRNA levels. TGF β 1 also induced the phosphorylation of ERK1/2. Importantly, Pre-treatment with MEK inhibitor U0126 inhibited both the induction of ERK1/2 phosphorylation and the up-regulation of integrin β 3 or α v by TGF β 1. Furthermore, siRNA knockdown and integrin α v β 3 antibody studies revealed that integrin β 3 and α v are required for TGF β 1-induced cell adhesion and migration. Our results

suggest that TGF β 1-MEK-ERK1/2-integrin α v β 3 signaling could contribute to poor patient survival of high risk endometrial cancer by promoting the adhesion and migration of cancer cells.

In chapter 5, we examined the relationship between TGF β 1 and PTEN, as well as the roles of PTEN in type II endometrial cancer migration. Treatment with TGF β 1 increased KLE and HEC-50 cell migration. Moreover, TGF β 1 treatment decreased PTEN mRNA and protein levels via both MEK-ERK1/2 and SMAD2/3-SMAD4 signalings. Importantly, overexpression of PTEN inhibited the basal and TGF β 1-induced cell migration. Additionally, PTEN down-regulation by TGF β 1 induced the activation of PI3K/AKT pathway and pharmacological inhibition of this pathway by Wortmannin and LY294002 abolished TGF β 1 induced migration. Our results suggest that TGF β 1-PTEN-PI3K/AKT signaling could contribute to poor patient survival by promoting the migration of type II endometrial cancers and can be promising therapeutic targets.

In summary, these results describe the functions of activin B and TGF β 1 in endometrial cancer migration or invasion or adhesion and the underlying novel molecular mechanisms. Activin B promotes the acquisition of invasive properties in type II endometrial cancer cells by up-regulating integrin β 3 and down-regulating SNAIL-mediated E-cadherin. TGF β 1 induces type II endometrial cancer cell migration by up-regulating integrin α v β 3 and down-regulating PTEN. Collectively, the results suggest therapies targeting activin and TGF β SMAD dependent and SMAD independent pathways may inhibit the migration of type II endometrial cancer and promote the survival of patients with this highly lethal cancer subtype.

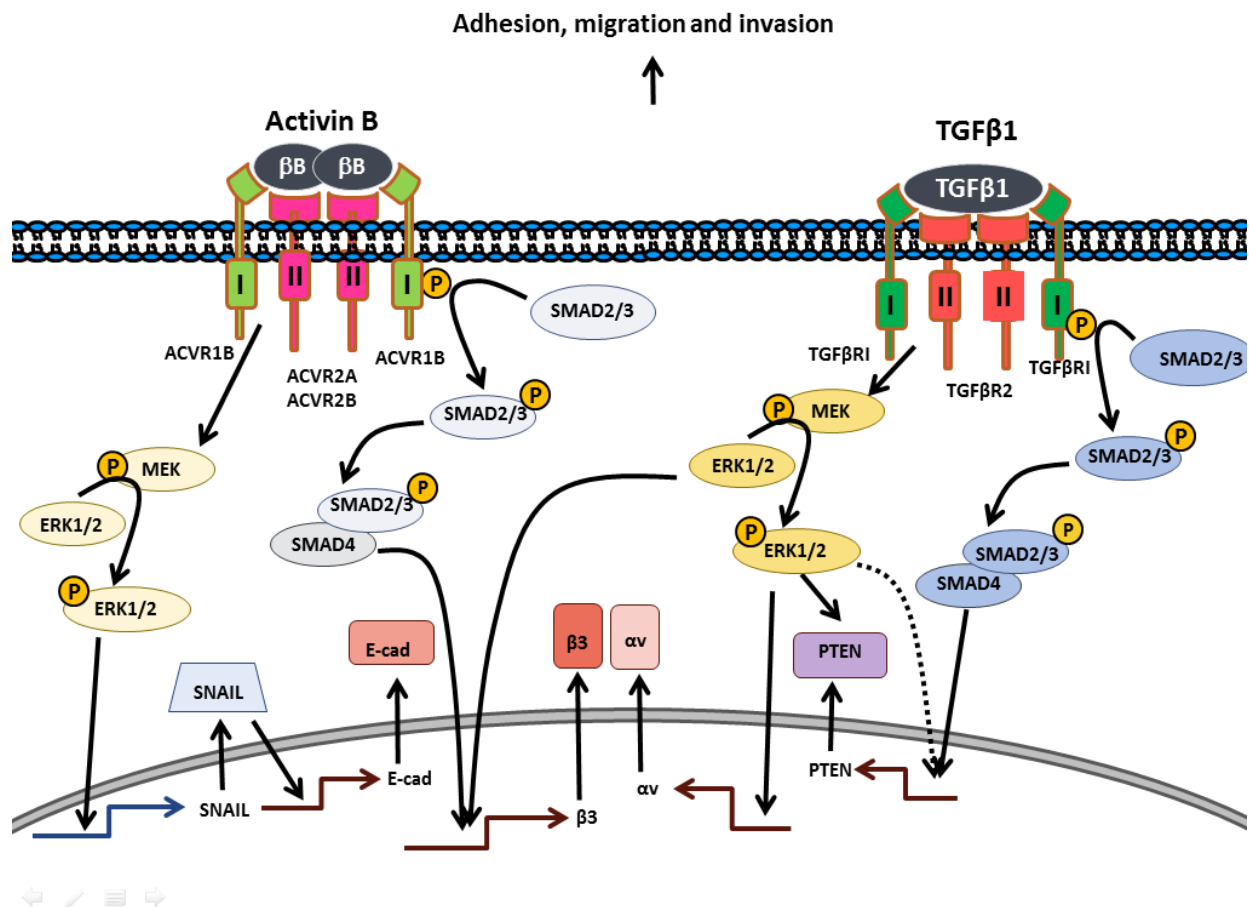


Figure 7.1 A schematic diagram of the proposed mechanisms of activating B and TGFβ1 regulating endometrial cancer cell adhesion, migration and invasion.

7.2 Overall discussion of this study

7.2.1 What are the similarities and differences between activin and TGFβ signalings? Is there any cross-talk between these two signaling pathways?

Our study shows the similarities and differences among activin signaling and TGFβ signaling. The similarities are: activin B and TGFβ1 can exert similar pro-oncogenic effects on endometrial cancer cells by potentiating their adhesion and migration ability; they both activate SMAD independent MEK-ERK1/2 and SMAD dependent signaling pathways; they can both target

integrin $\beta 3$ to increase the adhesion and migration of cancer cells. But meanwhile, they demonstrated disparities in the following aspects. They have different target gene: TGF $\beta 1$ can down-regulate PTEN while activin B can't. More interestingly, they use different signaling pathways to regulate the same target. Activin B drives SMAD2/3-SMAD4 signaling whereas TGF $\beta 1$ induces MEK-ERK1/2 to up-regulate integrin $\beta 3$. Actually, the similarities and disparities among activin and TGF β signalings have been documented in numerous literatures. Activins and TGF β s share similar structures, both of which form dimers by a disulfide bond. They all bind to heterodimeric receptor complexes consisting of type I and type II receptor subunits with serine/threonine kinase domains, which then initiate classical SMAD dependent signaling cascade that induce or repress transcriptional activity. Moreover, they also share the same SMAD-independent pathways including PI3K/AKT, MEK-ERK1/2 and Wnt/ β -catenin. Therefore, they induce similar effects in certain cell types. For examples, activin A and TGF β all promote wound healing by inducing angiogenesis, ECM deposition and inflammation. Similar to TGF β , activins act both as a tumor suppressor and a promoter in a context-dependent way [31]. At early stages of tumorigenesis, activin A and TGF β can induce cell cycle arrest via the cyclin-dependent kinase inhibitors such as p21 and p27 [31], whereas they both promote breast cancer cell invasion by regulating the microRNA-181 family [220] or promote cancer cell progression by mediating EMT [31]. However, activins and TGF β also differ in many aspects. They associate with different type I and type II receptors and can activate different downstream transcriptional factors. In human embryonic stem cells, activin A up-regulates Nanog while TGF β uses SRY-box2 to induces self-renewal and differentiation [31]. Activin A mainly acts as a potent anti-angiogenic factor while TGF β stimulates pro-angiogenic proteins and VEGF-A. TGF β s can suppress the invasiveness of extravillous cytotrophoblast cells by reducing MMP9,

whereas activin A promotes their invasiveness by inducing MMP2 [119]. They can also result in same consequences using divergent pathways. In advanced colon cancer, activin A uses PI3K/AKT pathway while TGF β uses MEK-ERK1/2 to induce SMAD4-independent migration and EMT [221]. Interestingly, in the same study, TGF β up-regulates p21 via SMAD4 dependent ERK signaling while activin A down-regulates p21 via SMAD4 independent PI3K/AKT pathway, with the net effect being p21 down-regulation. These evidences demonstrate differential and parallel activin and TGF β signaling in the same cell. When both signalings remain intact in the same cells, the net effects depend on whether activin signaling antagonizes or synergizes with TGF β signaling and which pathway plays a dominant role. Another study in this case is pre-treating endometrial carcinoma cells with activin A antagonized TGF β 1-induced growth inhibition effects in a dose-dependent way [222]. However, TGF β family can also interplay with other pathways including mitogen-activated protein kinase, phosphatidylinositol-3 kinase/AKT, Wnt, Hedgehog and Notch [223]. Therefore, it's critical to fully analyze the crosstalk between these pathways to minimize unwanted side effects.

7.2.2 What are functions and mechanisms of activin B and TGF β 1 or other growth factors in the type I endometrial cancer?

Our current study only focuses on type II endometrial cancer, mainly because of its much higher aggressiveness and lower survival rate. However, the effects of growth factors on the progression of type I endometrial cancer are also worth investigating. Currently, type I endometrial cancer is usually treated by surgery. However, around 30% of women diagnosed with endometrial cancer are younger than 54 years of age [224]. For these young women who want to preserve fertility, hormone therapy with progestin is conducted [225]. Unfortunately,

many hormone receptor-positive tumors fail to respond to progestins [226]. This might be explained by the crosstalk between growth factors and steroid hormone signaling pathways. Steroid hormones have been shown to directly or indirectly alter the expression of growth factors, their receptors, functions, and signaling pathway components [227], whereas growth factors can mediate hormone induced responses [228] or affect the expression of hormone receptor expression [229]. Since most type I endometrial cancers are hormone sensitive, clarifying the expressions, functions and mechanisms of growth factors as well as their interplay with steroid hormones will be important for developing endocrine or targeted molecular therapy for type I endometrial cancer and improve their living quality and preserve their fertility. However, so far the related studies are limited. The most commonly studied growth factor in type I endometrial cancer is IGF-1, with its receptor up-regulated and signaling hyper-activated in endometrial hyperplasia and cancer [230]. Moreover, IGF system promotes endometrial cancer progression and interacts with insulin, steroid hormones and other growth factors [231]. In endometrioid tumors, positive immunostaining for β B was correlated with higher grade, but not associated with overall, progression free or cause specific survival [105, 106]. Similarly, Kaplan-Meier analysis of endometrial cancers with endometrioid histology from TCGA ([11]; n=307) shows that samples with inhibin β B mRNA levels greater than the median are not significantly associated with reduced disease free survival (Log-rank $P = 0.163$, Figure 7A) or reduced overall survival (Log-rank $P = 0.428$, Figure 7B). These evidences suggest that the relationship between activin B overexpression and decreased survival may only be specific to type II endometrial cancer. The significance of inhibin β A remains controversial. While patients with a positive inhibin β A demonstrate significantly worse cause-specific survival when evaluated by staining intensity, there is no such relationship between inhibin β A immunoreactivity and survival when

using semi-quantification analysis. But both methods reveal that inhibin β A immunoreactivity was significantly associated with myometrial invasion [232]. Kaplan-Meier analysis of endometrial cancers with endometrioid histology from TCGA ([11]; n=307) reveals that samples with inhibin β A mRNA levels greater than the median are not significantly associated with reduced disease free survival (Log-rank $P = 0.335$) or reduced overall survival (Log-rank $P = 0.953$). However, immunoreactivity of activin A in tissue extracts, identified by ELIZA, was significantly higher in endometrioid cancer than in normal endometrium [233]. These evidences suggest complex roles of activin A in type I endometrial cancer progression. The immunoreactivity of TGF- β 1 is increased in the epithelial component of endometrial carcinomas compared with non-neoplastic tissues [37, 203] and the plasma level of TGF- β 1 is greater in Stage-Ib and Stage-Ic patients than in Stage-Ia patients [38]. These results suggest that TGF- β 1 may play a pivotal role in the endometrial carcinogenesis in an autocrine and/or paracrine manner. Regarding type I endometrial cancer, the levels of SMAD2 phosphorylation are weak or undetectable and the expression levels of type I and type II TGF- β receptors are decreased compared to matched normal tissues. Moreover, primary cultures of type I cancers do not response to the TGF- β 1-mediated growth inhibition [173]. These results suggest that TGF- β 1 classical signaling is disabled at an early stage of the tumorigenesis of type I cancers which lead to escape from normal growth control. However, the exact roles of functions and mechanisms of TGF- β 1 in type I endometrial cancer need further investigation.

7.2.3 What are the functions of activins and TGF β s in endometrial hyperplasia?

Endometrial hyperplasia is the excessive proliferation of endometrial cells. It's the precursor of type I endometrial cancer and associated with unopposed estrogen. It has been reported that up to

29% of untreated complex atypical hyperplasia progresses to carcinoma [234]. Progestin is the most commonly used treatment for endometrial hyperplasia, but around 30% of patients are progestin resistant [235]. Therefore, understanding the effects of growth factors on endometrial hyperplasia may help develop novel treatments, prevent its progression into endometrial cancer and shed some lights on the influence of these growth factors on endometrial carcinogenesis. Immunoreactivities of activin/inhibin β A and β B subunits have been demonstrated in endometrial hyperplasia and were significantly higher in hyperplasia than in cancer tissue [236]. Interestingly, there is a continuous decline of inhibin α from hyperplasia grade I to III and to endometrioid cancer [237]. Since two activin/inhibin β subunits lead to activins while one activin/inhibin β and inhibin α form inhibins, these evidences suggest that more activins rather than inhibins are formed with the progression of endometrial hyperplasia and may play critical roles in endometrial carcinogenesis. As for the TGF β s, evidences from a mouse model show that TGF β type I receptor deletion can enhance endometrial epithelial cell proliferation which ends in endometrial hyperplasia in aged females and up-regulate fibroblast growth factor 10 transcript levels [238]. Moreover, all three forms of TGF β s demonstrated a statistically significant stepwise increase in the expression from the normal proliferative endometrium to simple hyperplasia and on to complex hyperplasia [239]. Also, the immunoreactivities of TGF β receptors and phosphorylated SMAD2 are progressively decreased from moderate in hyperplasia to absent in endometrial cancer [240]. These evidences suggest intervention of TGF β signaling occurs at as early as premalignant hyperplasia stage. Therefore, study of endometrial hyperplasia and cancer can facilitate the finding of molecular targets for disruption of cancer cell growth inhibition escape and invasiveness enhancement regulated by growth factors.

7.3 Limitations of this study

The limitation of my study is that we didn't use animal models to confirm the results of these in vitro studies. For examples, we could block activin receptors, SMAD dependent or independent signaling pathway or target integrin $\beta 3$, E-cadherin, or PTEN by inhibitors or genetic knockdown and then transplant these tumor cells into animal models to observe their progression and development. If the results obtained from the in vivo study are similar to our in vitro studies, further preclinical and clinical studies could be carried out to investigate these potential therapeutic targets in type II endometrial cancer.

7.4 Future directions

- 1) Although immortalized cancer cell lines are homogenous, easily manipulated and propagated, and more likely to generate reproducible results, they cannot thoroughly represent the in vivo situation. Cancer cell lines are often derived from the same patient and thus cannot represent the heterogeneous population of clinical patients. Moreover, the lack of tissue architecture and tumor microenvironment may alter cell-cell interactions, signalings and tissue specific functions. Cells in culture also tend to acquire genotypic and phenotypic drifting. Therefore, cancer cell lines may behave quite differently from cells in vivo. To further confirm our results, researches based on human endometrial cancer tissues, primary cells from various endometrial cancer patients or animal models should be conducted in the future.
- 2) Type I endometrial cancer is prone to hormone regulations and therefore its carcinogenesis and therapy are all closely associated with estrogen or progestin functions. However, hormone signaling pathways have been suggested to crosstalk with growth

factors which results in different consequences. Therefore, the expression of growth factors in type I endometrial cancer tissues at protein or genetic levels should be studied. And the effects of relevant growth factors on type I endometrial cancer cell growth and migration as well as their interaction with hormones can be further investigated.

- 3) Cancer cell migration is a complicated process which involves cell-cell adhesion by cadherins and cell-extracellular matrix interaction by integrins. But it's also greatly affected by the degradation/activation of ECM mediated by MMPs. Therefore, the potential roles of MMPs in activins or TGF β -induced migration are worth studying.
- 4) Endometrium is a complicated system consisting of various cell types, hormones and growth factors. These different components can interact with each other. Therefore, studying the effects of other growth factors such as EGF, IGF, VEGF etc located at the endometrial cancer tissue and their interactions can provide a more comprehensive picture of the growth factor network regulating endometrial cancer progression and help identify the key molecular therapeutic targets.

7.5 Significance and translational potential

The present study is the first comprehensive research of the functions and underlying mechanisms of activin B and TGF β 1 in type II endometrial cancer, the most aggressive subtype. Immunohistochemical study or genomic analysis of clinical samples has implicated the critical roles of activin and TGF β signalings in the progression of endometrial cancer. This study further investigates the biological significance of these two signaling pathways in in vitro cell models of the aggressive type II endometrial cancer. Moreover, this study enhances our understanding of the roles of integrin α v, integrin β 3, E-cadherin, PTEN as well as the MEK-ERK1/2 signaling

and PTEN-PI3K/AKT signaling in activin B or TGF β 1-induced cancer cell migration. This detailed information may offer potential therapeutic targets and lead to the development of more specific approaches for treating advanced and resistant endometrial cancer.

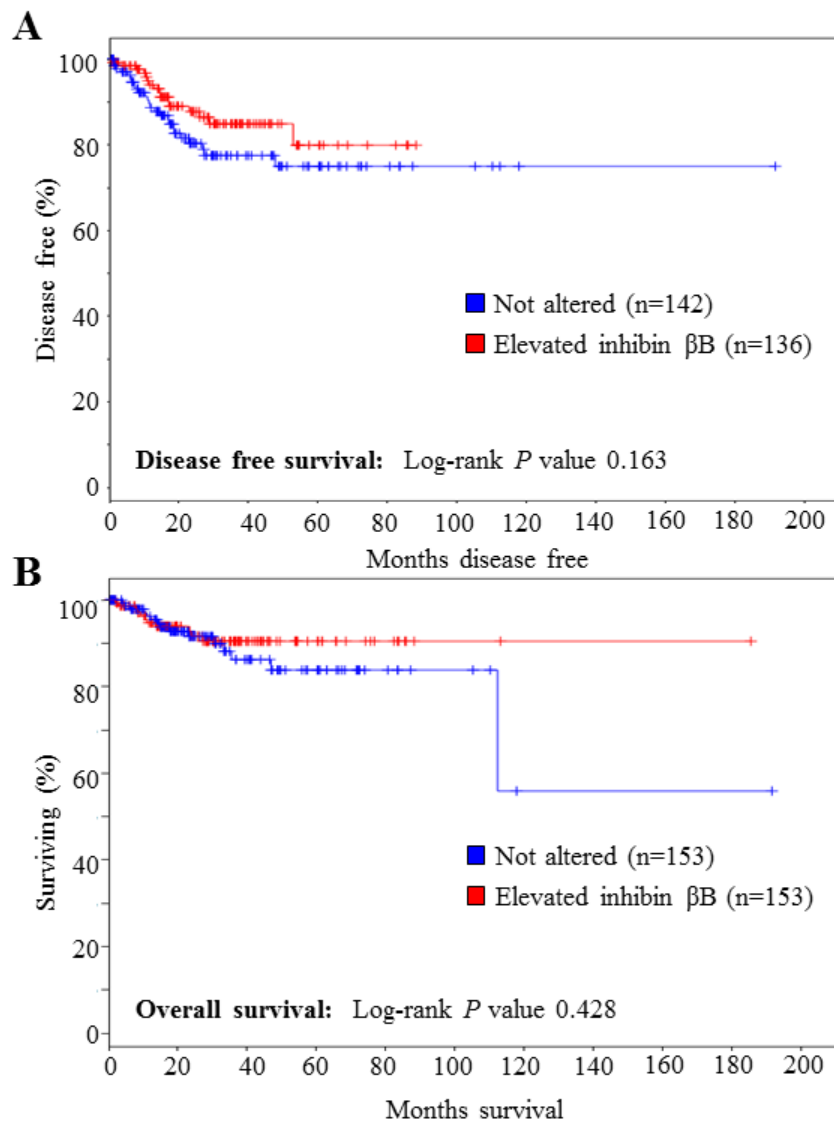


Figure 7. 2 Elevated inhibin β B is not associated with reduced disease free survival and a trend towards reduced overall survival in endometrioid endometrial cancers.

The cBioPortal for Cancer Genomics was used to query endometrial carcinomas with endometrioid histology from The Cancer Genome Atlas (n=307) for up-regulation of inhibin β B subunit mRNA above the median. Disease free (A) and overall (B) survival differences between unaltered samples and those with elevated inhibin β B are displayed as Kaplan-Meier survival curves with a P value from a Log-rank test.

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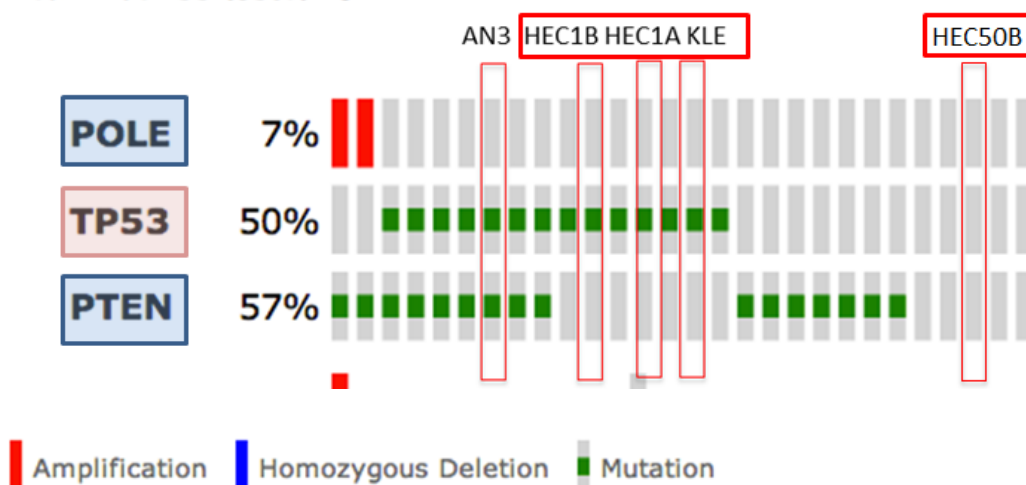
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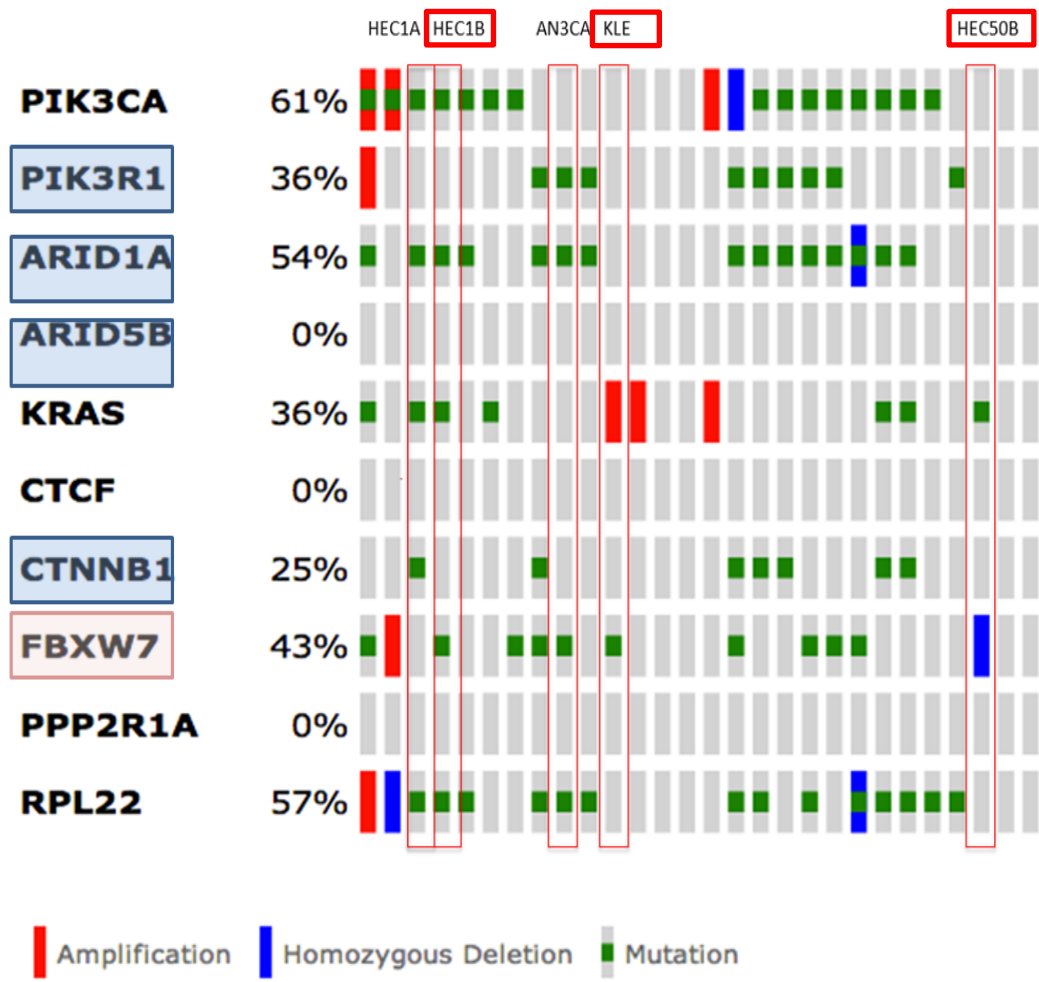
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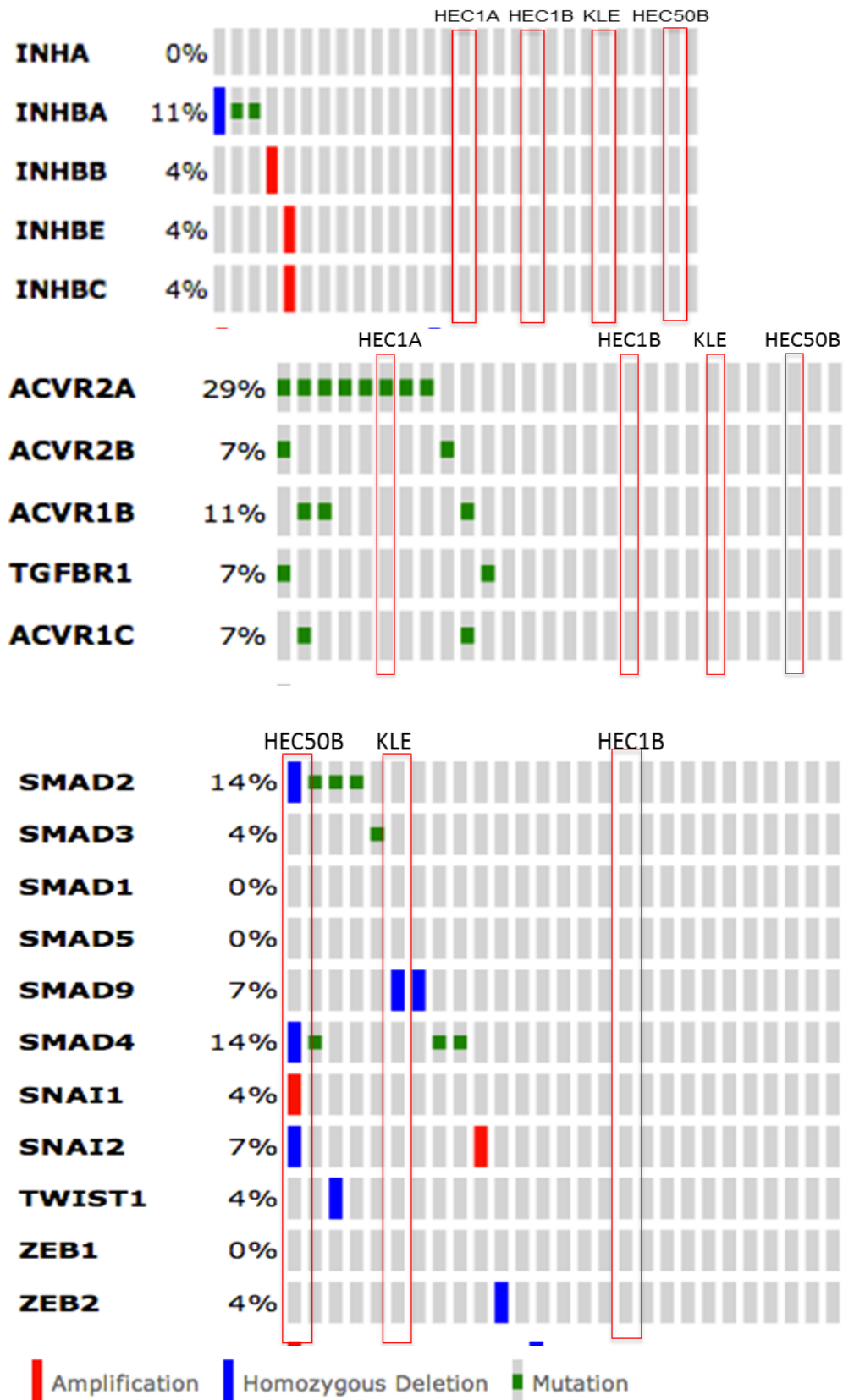
Appendix

Appendix A Endometrial Cancer Cell Lines Molecular Profiling

The genomic profiles of our cell models HEC1B, HEC50, KLE are extracted from Broad-Novartis Cancer Cell Line Encyclopedia (CCLE) in terms of DNA copy number alterations, mutations, and mRNA expression data. The investigated genes include typically mutated genes in endometrioid subtype (POLE, TP53, PIK3R1, ARID1A, ARID5B,CTNNB1) and serous like subtype (TP53, FBXW7), activin/inhibin subunits (INHA, INHBA, INHBB, INHBE, INHBC), activin receptors (ACVR2A, ACVR2B, ACVR1B, TGFBR1, ACVR1C), SMAD signaling molecules (SMAD2, SMAD3, SMAD1, SMAD5, SMAD9, SMAD4), and E-cadherin transcription factors (SNAI1, SNAI2, TWIST1, ZEB1, ZEB2).







Appendix B The mRNA expression of activating/inhibin subunits and receptors in various endometrial cancer cell lines

Total RNA of each endometrial cancer cell line was extracted with TRIzol Reagent (Invitrogen, Life Technologies) according to the manufacturer's instructions. Reverse transcription was performed using 2 g of total RNA, random primers and M-MLV reverse transcriptase (Promega, Fisher Scientific) in a final volume of 20 μ l. TaqMan RT-qPCR was performed on an Applied Biosystems 7300 Real-Time PCR System equipped with 96-well optical reaction plates. TaqMan gene expression assays for INHA, INHBA, INHBB, ACVR2A, ACVR2B, ALK4, ALK5, ALK7 were purchased from Applied Biosystems (Life Technologies). Each 20 μ l TaqMan RT-qPCR reaction contained 1 x Taqman Gene Expression Master Mix(Applied Biosystems), 50 ng cDNA, and 1 x specific Taqman gene expression assay containing primers and probe. Δ Cq was obtained by the mRNA expression level of each gene normalized to the mRNA expression level of GAPDH.

