

**THE ROLES OF BETACELLULIN IN EPITHELIAL OVARIAN CANCER  
MIGRATION AND PROLIFERATION**

by

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## **Abstract**

Epithelial ovarian cancer is the most lethal gynecological malignancy in developed countries. Although extensive studies have been done in the past decades to develop early detection methods and novel therapeutic strategies for epithelial ovarian cancer, 5-year survival rate of patients diagnosed in late stages remains lower than 30%. The epidermal growth factor (EGF) family of ligands and receptors is well-known for its roles in oncogenesis and cancer progression. As a unique member in this family, betacellulin has been shown to stimulate cell proliferation in several types of human tumors, and has also been suggested to associate with poor clinical outcome in breast cancer. However, little is known about the role and mechanism of betacellulin in epithelial ovarian cancer cell activities. In the current study, we hypothesized that betacellulin enhances epithelial ovarian cancer migration and proliferation by modulating several key target genes. Epithelial ovarian cancer cell lines OVCAR3, OVCAR4, OVCAR5 and SKOV3 were used as study models. Cancer cell migration was assessed by transwell assays; and cell proliferation was measured by MTT assays. Our results showed that betacellulin induced epithelial ovarian cancer cell migration by down-regulating E-cadherin and up-regulating Connexin43 expression; and these effects were mainly mediated by MEK/ERK and PI3K/Akt signaling via EGFR. Moreover, the potential role of ERBB4 in betacellulin signaling and ovarian cancer cell migration has also been suggested. On the other hand, we also demonstrated that betacellulin stimulated cell proliferation by up-regulating CCN1 expression. Taken together, our findings provide important insight into ovarian cancer biology and fill in the gaps of our knowledge of betacellulin, which may lead to the development of novel therapeutic tools for epithelial ovarian cancer.

## **Lay Summary**

Epithelial ovarian cancer is the most lethal gynecological malignancy and the survival of patients diagnosed with late stage disease remains very low (<30%). Betacellulin is a growth factor that has been shown to stimulate cancer cell proliferation and is associated with poor outcome in several cancers. The goal of this study was to investigate the role of betacellulin in epithelial ovarian cancer development and metastasis, in order to find new therapeutic targets or strategies for epithelial ovarian cancer. The results show that betacellulin induces epithelial ovarian cancer cell migration and proliferation. The involvement of several important target genes and signaling pathways in these processes was also confirmed. These findings provide important insights into ovarian cancer biology and increase our understanding of betacellulin, which may lead to the development of novel therapeutic tools for epithelial ovarian cancer.

## Preface

This study was approved by the University of British Columbia Children's and Women's Research Ethics Board (certificate no. H98-70175).

A version of chapter 3 has been published.

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I was responsible for the experimental design and conducted most of the experiments in this chapter. I analyzed the research data and wrote the manuscript, which was revised by Dr. Christian Klausen and my supervisor Dr. Peter Leung.

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## **List of Abbreviations**

ADAM: A disintegrin and metalloproteinase domain-containing protein

AKT: Protein kinase B

ANOVA: Analysis of variance

ARID1A: AT-rich interactive domain-containing protein 1A

BRCA1/2: Breast cancer 1/2

BSA: Bovine serum albumin

BTC: Betacellulin

CDH1: Cadherin-1

cDNA: Complementary deoxyribonucleic acid

CCOC: Clear cell ovarian cancer

CTNNB1: Catenin beta 1

DMSO: Dimethyl sulfoxide

dNTP: Deoxynucleoside triphosphate

ECL: Enhanced chemiluminescence

ECM: Extracellular matrix

EGF: Epidermal growth factor

EGFR: Epidermal growth factor receptor

EMT: Epithelial-mesenchymal transition

ENOC: Endometrioid ovarian cancer

EOC: Epithelial ovarian cancer

ERBB: Avian erythroblastosis oncogene B

ERK: Extracellular signaling-regulated kinase

FBS: Fetal bovine serum

FIGO: International Federation of Gynecology and Obstetrics

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

GJ: Gap junction

GJIC: Gap junctional intercellular communication

GRB2: Growth factor receptor bound-2

GTP: Guanosine-5'-triphosphate

HB-EGF: Heparin-binding EGF-like growth factor

HGSC: High-grade serous ovarian cancer

JNK: c-Jun N-terminal kinase

LGSC: Low-grade serous ovarian cancer

MAPK: Mitogen-activated protein kinase

MEK: Mitogen-activated protein kinase kinase

MET: Mesenchymal-epithelial transition

MMP: Matrix metalloproteinase

mRNA: Messenger ribonucleic acid

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MUOC: Mucinous ovarian cancer

NF- $\kappa$ B: Nuclear factor kappa-light-chain-enhancer of activated B cells

NRG: Neuregulin

OSE: Ovarian surface epithelium

PAGE: Polyacrylamide gel electrophoresis

PBS: Phosphate buffered saline

PI3K: Phosphatidylinositol 3-kinase

PIK3CA: Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha

PLC $\gamma$ : Phospholipase C

PPP2R1A: Protein phosphatase 2 scaffold subunit Aalpha

PTEN: Phosphatase and tensin homolog

RNA: Ribonucleic acid

RTK: Receptor tyrosine kinase

RT-qPCR: Reverse transcription quantitative real-time PCR

SD: Standard deviation

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM: Standard error of the mean

SHC: Src homology 2 domain containing transforming protein

siRNA: Small interfering RNA

STAT: Signal transducer and activator of transcription

STIC: Serous tubal intraepithelial carcinoma

TCGA: The Cancer Genome Atlas

TGF: Transforming growth factor

TP53: Tumor protein p53

VEGF: Vascular endothelial growth factor

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# Chapter 1: Introduction

## 1.1 Ovarian cancer

### 1.1.1 Overview

Ovarian cancer is the 5<sup>th</sup> leading cause of cancer-related death in women and the most lethal gynecological malignancy in developed countries [1, 2]. In 2016, it was estimated that 22,280 new cases and 14,240 deaths would occur in the United States [1]. In the past decade, the 5-year overall survival rate of ovarian cancer has increased from 36% to 46% due to improved surgical techniques combined with chemotherapeutic treatment [1, 3]. In spite of the good outcome in early stages (FIGO stages I and II), the 5-year survival rate of patients diagnosed in stage IV remains very low, approximately 25% [4].

One of the dominant reasons for the high lethality of ovarian cancer is the lack of efficient methods to detect the tumors early when they are still curable. Approximately 70% of patients are diagnosed at advanced stages (FIGO stage III and IV, Figure 1.1) with peritoneal implantation and distant metastasis, which makes it hard to be cured by cytoreductive surgery and easy to acquire chemo-resistance [5]. However, if the tumors can be detected when they are confined in the ovaries, the 5-year survival rate could be as high as 90% [6]. Accordingly, the high motility and invasive behavior of ovarian cancer cells are obstacles that need to be overcome to improve patient outcomes [7]. Under this circumstance, a better understanding of the mechanisms of ovarian cancer is required in order to develop more efficient therapeutic strategies and early diagnostic methods to improve the wellbeing of women with this disease.

### **1.1.2 Types of ovarian cancer**

Based on the anatomic structures from which the tumors originate, ovarian cancer can be classified into three major categories: epithelial ovarian cancers, sex-cord stromal tumors and germ cell tumors [8]. Epithelial ovarian cancers (EOC) were first thought to originate from ovarian surface epithelium (OSE). However, we now know that EOC has multiple cellular origins, most of which are derived from outside of the ovary, including fallopian tube epithelium and endometriosis [9]. EOCs account for more than 90% of ovarian cancers and comprise the majority of malignant ovarian cancers [10]. Sex-cord stromal tumors arise from mesenchymal and mesonephric origins. Approximately 7% of ovarian cancers are this type and they often combine with endocrine manifestations. Germ cell tumors account for only 3% of ovarian cancers. They are most common in children and young adults, compared to epithelial ovarian cancers whose patients are usually middle-aged or older [11].

### **1.1.3 Subtypes of epithelial ovarian cancer**

Epithelial ovarian cancer (EOC) is a heterogeneous disease which includes several subtypes. The four major subtypes, based on different histologic features and molecular abnormalities, are serous, endometrioid, clear cell and mucinous (Table 1.1) [10]. Among them, ovarian serous carcinomas can be further divided into high-grade serous carcinoma and low-grade serous carcinoma according to their clinical pathology and genetic characteristics [12].

High-grade serous carcinoma (HGSC) accounts for 70% of all EOCs and 90% of ovarian cancer deaths [13-15]. Consequently, HGSCs need to be focused on to significantly reduce the mortality and morbidity of EOC. *TP53* mutations are detected in more than 95% of cases [16], and inactivation of *BRCA1/2* also occurs in 40% to 50% of sporadic HGSCs [17]. Two key

reasons lead to the high mortality of HGSC. The first one is the advanced stage of the tumor at diagnosis. Less than 1% of cases are confined to the ovary upon diagnosis [18], and survival rates for patients with stage IIIc or IV disease are 29% and 13%, respectively [19]. The second is its highly chemoresistant nature. 70-80% of HGSCs are initially responsive to chemotherapy but the majority of tumors end up recurring as chemoresistant disease [20].

Clear cell ovarian carcinoma (CCOC) accounts for 4% to 12% of all EOCs in Western countries [21, 22] and has distinct clinical characteristics from other subtypes of EOC. The most common genetic changes in CCOC are mutations of *ARID1A* (in about 50% of cases) [23], *PIK3CA* (in about 50% of cases) [24], and *PTEN* (in about 20% of cases) [25]. Generally, CCOCs present at an early stage, especially stage Ic [26, 27]. But their prognosis is very poor (average survival time is 12.7–24.0 months), which may be due to their resistance to conventional platinum- or taxane-based chemotherapy [21, 22, 26, 28].

Endometrioid ovarian carcinoma (ENOC) accounts for approximately 10% of EOCs. It has a well-established association with endometriosis and shows similar morphological features to atypical hyperplasia of endometrial carcinoma [29]. Common genetic mutations in ENOCs are *CTNNB1* (in 38%–50% of cases)[29], *ARID1A* (in about 30% of cases)[30], and *PPP2R1A* (in about 12% of cases) [31]. ENOCs usually appear at both low-stage and low-grade, and have a favorable prognosis, although progression to high-grade carcinoma can occur [29].

Mucinous ovarian carcinoma (MUOC) accounts for only 2-4% of EOCs [32, 33] and is the least studied among all subtypes due to its rarity. Mutations of *KRAS* are detected in more than 75% of primary MUOCs [34-36], *ErbB2* gene alterations are also present in ~15% of cases [37-39]. The majority of MUOCs present at stage I and are associated with a favorable prognosis.

However, recurrent or metastatic MUOCs can appear at distant sites such as lung and bone, which will lead to poor prognosis [37, 40].

Low-grade serous carcinomas (LGSC) account for ~2% of EOCs and are thought to arise from borderline ovarian tumors [15]. Mutations of *KRAS*, *BRAF*, and *ErbB2*, which are involved in the RAS pathway, are found in the majority of these tumors [41]. In contrast to HGSCs, LGSCs are slow-growing, well-differentiated tumors that are generally diagnosed at a young age, present at low stage [42], and display poor responsiveness to conventional chemotherapy [43].

Over the last decades, a dualistic model of EOC has emerged based on the malignancy and genetic basis of its different subtypes. According to this model, EOC can be classified into two groups, type I and type II [33]. Type I tumors are usually detected at an early stage, progress slowly from well-established precursor lesions such as endometriosis and borderline tumor, and are characterized by mutations of *KRAS*, *BRAF*, *PTEN*, *ERBB2*, *ARID1A*, *PIK3CA*, *PPP2R1A* and/or *CTNNB1* [23, 30, 44]. LGSCs, low-grade ENOC, CCOC, MUOC and malignant Brenner tumor belong to this group. In contrast, Type II tumors are highly aggressive and usually present at advanced stages. They harbor *TP53* mutations in more than 95% of cases [45], and also have a high frequency of *BRCA1/2* mutation [16]. HGSC, high-grade ENOC and undifferentiated carcinoma belong to this type [44].

## **1.2 Epidermal growth factor (EGF) family**

### **1.2.1 Overview**

The EGF family is a group of peptide ligands that can bind to and activate receptors in the ERBB family [46]. A schematic representation of this family is shown in Figure 1.2. The ERBB family of receptor tyrosine kinases contains four members: EGFR (HER1/ERBB1), ERBB2

(HER2), ERBB3 (HER3) and ERBB4 (HER4) [47]. They are structurally-related and contain several domains: an extracellular domain for ligand-binding, a single transmembrane domain, an intracellular juxtamembrane domain followed by a tyrosine kinase domain, and a C-terminal which contains multiple tyrosine phosphorylation sites [48]. After ligand-induced activation, ERBB receptors form homo- or heterodimers and generate numerous cellular signals which are crucial for development, homeostasis and diseases [49]. It is worth noting that although ERBB2 cannot bind an EGF-like ligand, it is the preferred partner for other ERBB receptors and works as an amplifier for the network. Another exception is ERBB3 since it can strongly activate PI3K signaling despite lacking tyrosine kinase activity [47].

The EGF family of ligands comprises 13 members: EGF, transforming growth factor- $\alpha$  (TGF- $\alpha$ ), amphiregulin, heparin-binding EGF-like growth factor (HB-EGF), betacellulin (BTC), epiregulin, epigen, neuregulin-1 (NRG-1), NRG-2, NRG-3, NRG-4, NRG-5 and NRG-6 [49]. They are first produced as glycosylated transmembrane proteins and then cleaved to release mature growth factors. The EGF family can be further divided into three groups based on their binding properties [50, 51]. The first group comprises EGF, TGF- $\alpha$ , epigen and amphiregulin, which exclusively bind to EGFR. Epiregulin, BTC and HB-EGF belong to the second group and they can bind to both EGFR and ERBB4. NRGs make up the third group which binds to ERBB3 and/or ERBB4.

### **1.2.2 EGFR structure and signaling**

EGFR is the prototypical receptor tyrosine kinase (RTK). The extracellular domain of EGFR contains two homologous ligand binding subdomains (domains I and III) and two cysteine rich subdomains (domains II and IV) [48]. Before ligand-binding, domain II is buried by

intramolecular interaction with domain IV; this event autoinhibits the contact between two receptors. When a ligand binds to domain I and III, the extracellular region changes to an extended configuration, and two receptor molecules dimerize through direct interactions with domain II [48, 52]. Following receptor dimerization, several adaptor proteins are recruited to the phosphorylated tyrosine residues of EGFR, such as growth factor receptor bound-2 (GRB2), Src homology 2 domain containing transforming protein (SHC), signal transducer and activator of transcription (STATs) and phospholipase C (PLC $\gamma$ ). These proteins in turn recruit downstream signaling molecules which trigger multiple signaling pathways, including Ras-MAPK and PI3K-Akt pathways [47, 53, 54].

### **1.2.3 EGF family in cancer**

EGF-like growth factors and ERBB receptors play a central role in pathogenesis and cancer progression. Overexpression of these proteins has been widely identified in the majority of human tumors. Specifically, EGFR or ERBB3 expression has been found in 50% to 70% of lung, colon and breast carcinomas. ERBB2 is expressed in 30% of breast cancers, while ERBB4 is expressed in more than 50% of breast and ovarian tumors [55]. More importantly, overexpression of EGFR or ERBB2 has been shown to indicate early relapsing and poor prognosis in breast cancer patients [56, 57]. In addition to the high frequency of expression of individual receptors, co-expression of several ERBB receptors has been shown to associate with more aggressive phenotype and worse outcome in invasive breast cancers, lung cancers, colon cancers and oral squamous cell cancers [58-60]. Co-expression of EGF-like growth factors, such as TGF- $\alpha$ , amphiregulin and NRGs, has been demonstrated in lung, breast, colon and gastric

carcinomas, which suggests the importance of the integrated network of EGF family in oncogenesis and cancer progression [61].

#### **1.2.4 Betacellulin (BTC)**

Betacellulin (BTC) is a member of the EGF family of growth factors and is encoded by the *BTC* gene on chromosome 4 in humans [62]. It is first produced as a 178 amino acid long membrane-anchored precursor, and then undergoes ectodomain shedding modulated by a disintegrin and metalloprotease (ADAMs) to release the mature BTC protein. BTC has been classified as “pan-ERBB ligand” since it can directly bind to both EGFR and ERBB4, and activate all the possible combinations of ERBB dimers [63]. Following receptor activation, BTC can trigger numerous intracellular signaling pathways, including MAPK, PI3K-Akt and JNK, and modulate crucial cell activities, such as cell growth, proliferation and motility [64-66].

BTC was first identified in pancreatic  $\beta$ -cell tumors as a mitogen [67]; later studies showed that it can strongly induce  $\beta$ -cell proliferation and improve glucose metabolism [68-70]. BTC has also been found to stimulate keratinocyte proliferation [71], and enhance angiogenesis by inducing growth and migration of different types of vascular smooth muscle cells [72]. In female reproduction, rapid and transient up-regulation of BTC triggered by luteinizing hormone (LH) is important for cumulus expansion and oocyte maturation [73]. Expression of BTC combined with EGFR in blastocyst apposition also suggests its involvement in embryo implantation [74].

The potential roles of BTC in cancer have been investigated in several studies. Overexpression of BTC has been identified in pancreatic cancer [75], endometrial cancer [76] and liver cancer [77]. BTC has also been shown to stimulate pancreatic cancer cell growth [78], and induce head and neck squamous cell carcinoma cell invasion by up-regulating matrix metalloproteinase

(MMP9) [79]. In breast cancer, BTC expression was associated with poor clinical outcome [80]; and more importantly, the “pan-ERBB ligand” property of BTC may account for the resistance of breast cancer cells against EGFR inhibitors [81]. Taken together, BTC plays multiple roles in physiological and pathological processes, and its mechanisms of action may differ from those of other EGF family members due to its unique binding properties. There are still large gaps in our knowledge about this important growth factor, and its roles in cancer progression and underlying mechanisms are worth studying in order to find more efficient therapeutic strategies.

### **1.3 Epithelial-mesenchymal transition (EMT)**

#### **1.3.1 Overview**

Epithelium and mesenchyme are two types of animal tissues. They have different appearance and characteristics, and constitute unique multicellular structures which possess different functions. Epithelial cells have regular shapes, usually squamous, cuboidal or columnar. They are closely held together by cell junction and adhesion structures between adjacent cells; and their basal surfaces normally adhere to basement membrane. These properties make sure that epithelial cells are arranged in uniform array like a sheet and limit the movement and motility of single cells. On the other hand, mesenchymal cells usually have spindle-like shape and front-to-back leading edge polarity. They lack cell-cell adhesions and uniform array, which allows for increased migratory capacity of mesenchymal cells [82]. Epithelial-mesenchymal transition (EMT) is a crucial biological process that allows epithelial cells to lose cell-cell junctions, change their polarity, rearrange their cytoskeleton, and exhibit mesenchymal phenotype, which includes enhanced motility and invasiveness [83, 84]. EMT was first identified in embryonic development and morphogenesis, and the reverse process of mesenchymal-epithelial transition

(MET) is also important in organ development [85]. For the past few years, studies have shown that EMT is also important for tumor progression. Epithelial tumor cells gain mesenchymal phenotypes during EMT and escape from the primary tumor, which is the first step of metastatic dissemination [86].

EMT is generally classified into three subtypes due to their different bio-functional consequences [87]. Type I EMT is associated with embryonic development, implantation and tissue morphogenesis. The main outcome of this kind of EMT is generating primary mesenchymal cells that can subsequently change into secondary epithelia. Type II EMT is often seen in wound healing and tissue regeneration. Fibroblasts and other related cells are produced in response to trauma or inflammation in order to repair the tissues. However, ceaseless response to these inflammatory signals will also lead to fibrosis and eventually organ destruction [88]. Type III EMT is important for cancer progression and metastasis. Cancer cells undergoing type III EMT acquire migratory and invasive abilities and eventually lead to the life-threatening stage of cancer [89].

EMT and EMT-like phenotypes are modulated by five main pathways [90, 91]: tyrosine kinase receptors (e.g. epidermal growth factor), integrins, Wnt, NF- $\kappa$ B, and TGF- $\beta$  pathways. Transcriptional regulation of junctional components (e.g. E-cadherin) and their transcription factors has also been shown to cause or be a result of EMT. Down regulation of E-cadherin and up-regulation of N-cadherin has been considered the hallmark of EMT [92].

## 1.3.2 E-cadherin

### 1.3.2.1 Overview

Cadherins are a large family of calcium-dependent glycoproteins that comprise over 100 members in humans. They are one of the major components of adherens junctions and are widely associated with cell adhesion, morphogenesis, recognition and tissue integrity [93]. Cadherins are type I transmembrane proteins, each contains an extracellular region, a transmembrane domain and a cytoplasmic tail. Cadherins on neighboring cells undergo homophilic interaction through their extracellular cadherin domains. Their cytoplasmic domain interacts with p120 catenin and  $\beta$ -catenin;  $\beta$ -catenin in turn binds to  $\alpha$ -catenin, and the latter participates in actin-containing cytoskeleton regulation [93, 94]. This cadherin-catenin complex is one of the core structures for maintaining cell adhesion.

Cadherins can be classified into several groups [95]. E-cadherin, which is the most-studied member of this superfamily, belongs to the type I (or classical) cadherins [96]. E-cadherin is encoded by tumor suppressor gene *CDH1* and is mainly expressed by epithelial cells in the region of cell-cell contact [97]. Mature E-cadherin contains five extracellular cadherin repeats and forms homodimers to interact with homodimers on adjacent cells in the presence of  $\text{Ca}^{2+}$  [98]. E-cadherin is very important for epithelia formation, cell morphology and maintenance of polarity. Mutations in the *CDH1* gene or down-regulation of E-cadherin via other mechanisms affects cell-cell adhesion, decreases epithelia integrity and increases cell motility, which may lead to oncogenesis and tumor metastasis [98, 99]. Besides its role in maintaining normal adherens junctions, E-cadherin negatively regulates the canonical Wnt pathway which can modulate the EMT process [100]. In general, loss of E-cadherin is one of the most crucial events in EMT and is associated with epithelial tumor metastasis [82].

### 1.3.2.2 Regulation of E-cadherin

E-cadherin expression can be dysregulated at various levels in human tumors. On the genetic level, mutations in the *CDH1* gene can cause abnormal expression of E-cadherin protein and loss of functional cell-cell adhesion. Loss of heterozygosity, specific inactivating mutations and germline mutations in *CDH1* gene have been found in several types of human cancers [101-104]. In addition to genomic mutations, epigenetic silencing of the *CDH1* gene promoter region also suppresses E-cadherin expression [105]. On the transcriptional level, several transcription factors bind to E-boxes located in the *CDH1* promoter and strongly suppress E-cadherin. These repressors include Snail (SNAIL1) and Slug (SNAIL2) from the snail family, Twist (TWIST1) from the basic helix-loop-helix (bHLH) family, and ZEB1 and ZEB2 from the zinc finger homeobox family [106]. Furthermore, phosphorylation, glycosylation and other modifications after transcription are also very important in modulating E-cadherin expression [107].

The Snail superfamily of zinc-finger transcription factors occupies a central role in cell movements, which occur in both morphogenesis and tumor progression [108]. It can be subdivided into two related but independent groups, the Snail and the Scratch families [109]; and the vertebrate *Snail* genes can be further divided into two subfamilies, Snail and Slug. The transcription factors encoded by Snail family members share a similar structure: a divergent N-terminal region; and a highly conserved C-terminal region which contains four to six zinc fingers that can bind to E-box elements on Snail-related genes. Snail and Slug play crucial roles in triggering EMT during embryonic development. Antisense Slug RNA injection led to Slug reduction and defects in neural crest migration and mesoderm delamination [110, 111]. E-cadherin, which is the most commonly studied target for Snail family members, is directly repressed by Snail or Slug in mesoderm development and epithelial tumors [112, 113].

### **1.3.2.3 E-cadherin in cancer**

EMT is a key step for tumor cells to escape from the primary tumor mass, disseminate and metastasize. Suppression of E-cadherin by its transcriptional repressors, such as Snail and Slug, is a crucial event driving EMT. Inducers of EMT, including EGF, TGF- $\beta$ , hepatocyte growth factor, Wnt and Notch signaling, which are produced by the tumor microenvironment or the tumor cells themselves, can trigger the expression of those transcriptional repressors and inhibit E-cadherin expression, which will further contribute to the malignant progression of various human tumors [90, 100].

The metastatic pattern of ovarian tumors is quite different from other epithelial tumors. Ovarian tumors can directly extend to adjacent organs, and dispersed tumor cells can be transported by normal peritoneal fluid throughout the peritoneal cavity. This unique nature is partially attributed to anatomy and may also depend on the loss of E-cadherin and cell-cell adhesion during EMT [7]. In ovarian tumors, the levels of E-cadherin are much higher in benign and borderline ovarian tumors compared to malignant adenocarcinomas as shown using an immunohistochemical technique [114]. Furthermore, reduced expression of E-cadherin is significantly correlated with poor overall survival, poor differentiation and serous histological type [115, 116]. In murine ovarian tumor cells, E-cadherin levels are negatively correlated with cell metastatic abilities [117]. In addition, knockdown of endogenous E-cadherin has been shown to promote human ovarian cancer cell invasion [118]. Our previous studies also demonstrated that EGF can promote ovarian cancer invasion by down-regulating E-cadherin expression via different pathways [119-121]. Taken together, E-cadherin and EMT play important roles in ovarian cancer metastasis, and a better understanding of the factors and mechanisms modulating E-cadherin expression in ovarian cancer could provide new insights for treating this devastating disease.

## **1.4 Connexin43 (Cx43)**

### **1.4.1 Cell junctions**

In multicellular organisms, epithelial cells closely connect with each other and attach to ECM to provide the lining and barrier function of epithelium. Four types of cell-cell junctions mediate the connection and communication between epithelial cells: tight junctions, adherens junctions, desmosomes and gap junctions [122, 123]. Tight junctions are distributed at the apical region of the cell, sealing the gap between lateral cells thereby creating a barrier between different parts of body. Tight junctions also serve as paracellular gates and only let molecules with specific size and charge to pass through [124]. Adherens junctions and desmosomes are anchoring junctions; they link cytoskeleton actin filaments and intermediate filaments between adjacent cells [122]. Adherens junctions also work with tight junctions to make up the apical junctional complex and form the border of apical and basolateral membrane in highly polarized epithelial cells [93, 124].

### **1.4.2 Gap junction (Gj)**

Gap junctions (Gj) are channels between adjacent cells that allow direct communication and transportation of molecules smaller than 1kDa, such as ATP, ions, small metabolites and second messengers [125]. They are assembled from two connexons which are embedded in the plasma membrane of adjacent cells. Connexon, also known as connexin hemichannel, is composed of six connexin subunits. Each connexin subunit is a four-pass transmembrane protein containing two extracellular loops, one cytoplasmic loop, one cytoplasmic N-terminal domain and one C-terminal tail which includes binding sites for structural and signaling molecules. Highly conserved residues in the extracellular loops are critical for the docking of two hemichannels and

contribute to cell-cell adhesion [126]. Humans have 21 connexin genes, which are expressed in a cell type-dependent manner yielding a great variety of functions [127].

Molecular exchange through gap junctions is essential for signal transduction and propagation in cells and contributes to numerous physiological activities such as embryonic development, growth control, cell apoptosis and differentiation [128]. Undocked hemichannels also have functional roles since they allow exchange of molecules and ions between cells and their environment. Signaling molecules (e.g. PGE<sub>2</sub>, ATP and NAD<sup>+</sup>) released from hemichannels act in both autocrine and paracrine manner to induce multiple signaling responses [129]. Hemichannels also take in small metabolites (e.g. glucose) and transport second messengers (e.g. Ca<sup>2+</sup>) which are crucial for cell biology [129]. Opening and closure of gap junctions and hemichannels are precisely modulated by several mechanisms, such as change in voltage, growth factors and connexin post-transcriptional modifications [130]. Mutations of connexin genes or abnormal expression of connexin isoforms leads to malfunction of gap junction communication and are involved in many diseases and pathologies [125, 131-133]. Mutations in *GJA3* (Cx46) and *GJA8* (Cx50) cause cataracts [134, 135]; *GJB1* (Cx32) mutations cause myelin-related disease [136]; while hearing loss and skin diseases are caused by mutations in *GJB2* (Cx26), *GJB3* (Cx31) and *GJB4* (Cx 30.3) genes [137-139]. Besides genetic mutations, aberrant expression of connexins also leads to several disorders. For instance, altered expression patterns of vascular connexins (Cx37, Cx40 and Cx43) have been identified in atherosclerotic plaques [140]. In recent years, the roles of connexins independent of their channel activities have been increasingly studied. It is reported that connexins can modulate cell growth, adhesion, migration and apoptosis in a gap junctional intercellular communication (GJIC)-independent manner [131, 141, 142].

### 1.4.3 Connexin43

Connexin43 (Cx43) is the most studied and widely expressed member of the connexin family [143, 144]. It is encoded by the *GJA1* gene and its C-terminal tail is the primary region that transduces multiple signaling pathways upon phosphorylation and also provides sites for protein-protein interaction. Activation of protein kinase A and casein kinase 1 phosphorylates Cx43 and increases its movement to plasma membrane and assembly into gap junction plaques [145, 146]. On the contrary, activation of protein kinase C [147], and MAPK [148] by growth factors (e.g. EGF) or phorbol esters affect Cx43 half-life and cause Cx43 internalization, which disassembles gap junctions and inhibits GJIC. Of note, binding of src and phosphorylation of Cx43 by src can activate at least src, protein kinase C and MAPK pathways and down-regulate gap junction; this mechanism is used by several ligands and receptors to inhibit GJIC, including G protein-coupled receptors [149] and tumour necrosis factor- $\alpha$  [150].

Besides interacting with kinases, Cx43 can also crosstalk with other signaling proteins and scaffold molecules, which lead to the changes in GJIC and cell activities. Zona occludens-1 (ZO-1), which is a tight junction-related protein, has been shown to directly bind with Cx43 and may serve to anchor cytoskeleton and recruit signaling proteins to Cx43-based gap junctions [151, 152]. N-cadherin and  $\beta$ -catenin have been shown to co-localize with Cx43 in cardiac myocytes [153, 154]; and Cx43 is thought to sequester  $\beta$ -catenin at gap junctions thereby modulating Wnt-mediated gene expression [154]. In addition, Cx43 can directly bind with  $\alpha$ - and  $\beta$ -tubulin [155], and also co-localizes with lipid raft protein caveolin-1 [156] and ECM protein NOV/CCN3 [157].

Cx43 plays crucial roles in tissue homeostasis, cardiovascular functioning, nervous system development, wound healing and embryogenesis [143]. Mutation or aberrant expression of Cx43 leads to a broad spectrum of disorders. Oculodentodigital dysplasia, a rare autosomal genetic

disease characterized by facial, dental and digital anomalies, is caused by *GJA1* mutation [158-160]. In the cardiovascular system, *GJA1* mutation is responsible for heart malformations and defects of laterality [161-163]; and mice with *GJA1* null mutation also lost the heart protection from ischemic preconditioning [164, 165]. In skin homeostasis, Cx43 expression levels are decreased during wound healing, and continuously elevated expression of Cx43 in diabetic skin markedly delays wound closure [166, 167].

#### **1.4.4 Connexin43 in cancer**

Cx43 was initially considered to be a tumor-suppressor since reduced expression of Cx43 was observed in several types of human cancer [168, 169]. In breast cancer, Cx43 was shown to be reduced at various stages of progression and in cell lines [170]. HeLa cervical carcinoma cells also lack Cx43 expression, and forced-expression of Cx43 reduces the neoplastic phenotype of these cells [171]. Similar patterns of reduced Cx43 expression have been observed in lung, prostate and Leydig cell tumors [172-174]. In ovarian cancer, the expression of Cx43 was barely detectable when compared to normal ovarian epithelial cells which have moderate expression [175]. Our previous study also showed that EGF-induced Cx43 negatively regulates ovarian cancer cell proliferation [176]. However, growing evidence indicates the positive involvement of Cx43 in oncogenesis and cancer progression. Cytoplasmic expression of Cx43 was shown to increase progressively in colon cancer [177]. Moreover, Cx43 facilitated breast cancer metastasis and melanoma brain colonization [178]. We have also found that Cx43 mediates the TGF- $\beta$ -induced increases in ovarian cancer cell migration [179]. Taken together, the roles of Cx43 in cancer progression are complex and a better understanding of the molecular mechanisms

underlying the roles of Cx43 will provide us with new therapeutic strategies for the prevention and treatment of Cx43-related diseases.

## **1.5 Cysteine-rich protein 61 (CYR61/CCN1)**

### **1.5.1 CCN family overview**

The CCN family is a group of extracellular matrix (ECM) proteins that modulate numerous biological activities and functions in humans, including embryonic development, wound healing, angiogenesis, fibrosis and oncogenesis [180]. This family comprises six members: cysteine-rich protein 61 (CYR61/CCN1), connective tissue growth factor (CTGF/CCN2), nephroblastoma overexpressed (NOV/CCN3), Wnt-1-induced secreted protein-1 (WISP-1/CCN4), WISP-2/CCN5, and WISP-3/CCN6 [181]. Most of them share four conserved functional domains and one non-conserved central hinge region that determines their respective binding partners [182]. The preferred receptors for the CCN family are integrins, including  $\alpha_v\beta_3$ ,  $\alpha_v\beta_5$ ,  $\alpha_5\beta_1$  and  $\alpha_6\beta_1$ . CCNs also bind with high affinities to heparin sulfate proteoglycans, low density lipoprotein receptor-related proteins, and mannose-6-phosphate receptor [182]. Notably, the utilization of receptors by CCNs is cell type- and function-specific. CCNs can also elicit numerous bioactivities by interacting with a wide range of growth factors, including insulin-like growth factors, TGF- $\beta$ , bone morphogenetic proteins and vascular endothelial growth factor (VEGF) [181]. The complex interaction between CCNs and their binding partners activates diverse signaling pathways and further contributes to the versatility of cell responses, including cell adhesion, migration, invasion, proliferation and survival [183-188].

Physiologically, CCNs are associated with embryogenesis, angiogenesis and wound healing. CCN1 or CCN2 knockout mice exhibit aberrant vascular or lung development [189, 190]; and

CCN3 and CCN4 were shown to modulate skeletal growth and cardiac development [191, 192]. Increased expression of CCN1 and CCN2 has been observed during injured tissue repair, wound healing and after ischemia or bone fracture [193-195]. In cancer biology, aberrant expression of CCNs has been observed in many malignant tumors, including those of the breast, colon, liver and lung [181]. CCNs have also been implicated in oncogenesis, dissemination/metastasis, and prognosis [181, 196-199]. Interestingly, individual CCN members may have dualistic roles within a given cancer or even opposite roles in different types of cancer [181]. Such complexity is likely to stem from the specific binding between CCNs and different binding partners (e.g. integrins) in different cell types.

### **1.5.2 CCN1 functions and signaling**

CCN1 is the first member identified in the CCN family. It is a growth factor-inducible, immediate-early gene product and was initially described to modulate cell proliferation in fibroblasts [200]. Now we know that CCN1 is incredibly versatile, it regulates multiple cell behaviors including proliferation, adhesion, migration and apoptosis, and plays important roles in embryogenesis, angiogenesis and tumorigenesis [201]. CCN1 exerts its functions by binding with five integrins ( $\alpha_v\beta_3$ ,  $\alpha_v\beta_5$ ,  $\alpha_6\beta_1$ ,  $\alpha_{IIB}\beta_3$  and  $\alpha_M\beta_2$ ) and co-receptor heparin sulfate proteoglycans, and activates several types of signaling pathways including Wnt, NF- $\kappa$ B, ERK MAPK and PI3K/Akt [201]. Many stimuli modulate the production of CCN1 including growth factors (EGF, platelet-derived growth factor, VEGF and TGF- $\beta$ ), cytokines (interleukins and tumor necrosis factor- $\alpha$ ) and even mechanical stretch and inflammation [201]. EGF was shown to up-regulate CCN1 expression via JAK2/STAT3 signaling in endometrial epithelial cells [202]; whereas RhoA GTPase and p38 MAPK signaling mediates sphingosine 1-phosphate-induced

CCN1 expression in smooth muscle cells [203]. CCN1 is also a direct target of canonical Wnt/ $\beta$ -catenin signaling in osteoblast differentiation [204].

### **1.5.3 CCN1 in cancer**

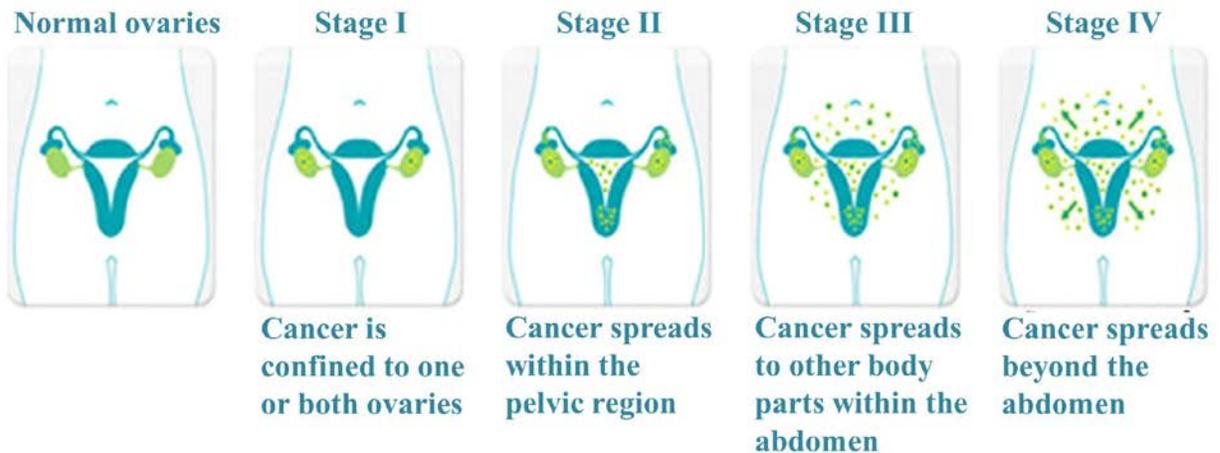
Several studies have demonstrated the involvement of CCN1 in oncogenesis and cancer prognosis. Overexpression of CCN1 has been extensively observed in multiple human tumors, including breast cancer, prostate cancer, glioma, endometrial cancer and colon cancer [205]. Furthermore, CCN1 overexpression is associated with breast cancer oncogenesis, migration and invasion, and CCN1 can protect cancer cells from chemotherapy-induced apoptosis [206-208]. In addition, CCN1 serves as a prognostic marker because high CCN1 expression has been shown to be associated with advanced stage, poor differentiation and poor survival in glioma, liver cancer and colon cancer [209-211].

In human ovarian tumors, CCN1 expression is significantly higher in epithelial ovarian cancer, especially in HGSC, compared to benign ovarian tissue [209, 212]; and overexpression of CCN1 is markedly associated with advanced FIGO stage and poor prognosis [213]. Previous studies have suggested the involvement of several potential signaling pathways: CCN1 enhances ovarian cancer growth via PI3K/Akt and ERK MAPK signaling pathways; and inhibits cisplatin-induced apoptosis by modulating p53 and NF- $\kappa$ B expression [214-217]. Our recent study shows that sphingosine 1-phosphate induces CCN1 expression by disrupting Hippo pathway signaling, which further contributes to ovarian cancer cell proliferation [218]. Taken together, CCN1 likely plays crucial roles in ovarian cancer growth and progression, and a greater understanding of its activities including its upstream and downstream interactions could be helpful in identifying novel molecular targets to better manage this fatal disease.

**Table 1.1 Histologic subtypes of epithelial ovarian cancer.**

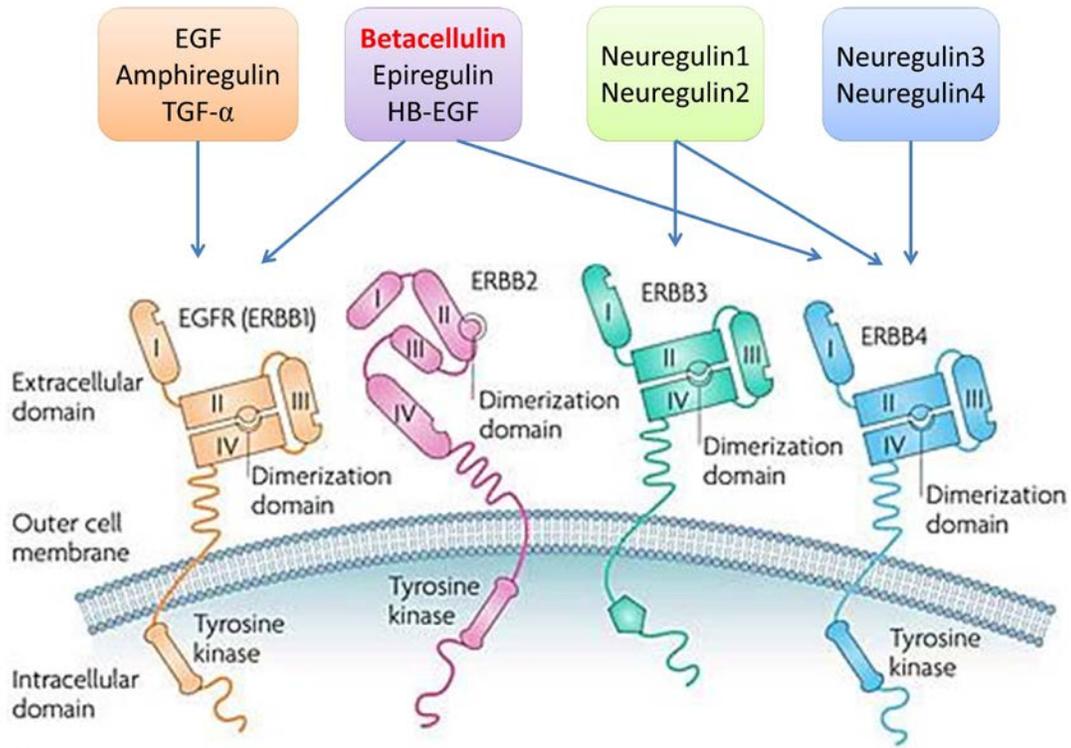
	<b>HGSC</b>	<b>LGSC</b>	<b>EC</b>	<b>CCC</b>	<b>MC</b>
<b>Precursor Lesions</b>	STIC	SBOTs	Endometriosis	Endometriosis	Unidentified
<b>Molecular Abnormalities</b>	<i>TP53</i> ; <i>BRCA1/2</i> ; chromosomally unstable	<i>BRAF/KRAS</i> chromosomally stable	<i>PTEN</i> ; <i>ARID1A</i> ; $\beta$ -catenin; microsatellite instability	<i>PI3CA</i> ; <i>KRAS</i> ; <i>PTEN</i> ; <i>ARID1A</i> ; microsatellite instability	<i>KRAS/HER2</i>
<b>Response to chemotherapy</b>	High	Intermediate	High	Low	Low
<b>Prognosis</b>	Poor	Intermediate	Favorable	Intermediate	Favorable

HGSC: High-grade serous carcinoma; LGSC: Low-grade serous carcinoma; EC: Endometrioid carcinoma; CCC: Clear cell carcinoma; MC: mucinous carcinoma; SBOT: Serous borderline tumor; STIC: Serous tubal intraepithelial carcinoma



**Figure 1.1 FIGO stages of ovarian cancer.**

This figure is adapted from <http://www.deepameditours.com/treatments/ovarian-cancer-cancer-of-the-ovaries/>



**Figure 1.2 Schematic depicting EGF family of ligands and receptors.**

This figure is adapted from Baselga J, Swain SM. Nat Rev Cancer. 2009 Jun 18;9(7):463-75.

## **Chapter 2: Rationale and objectives**

### **2.1 Rationale**

Ovarian cancer is the most lethal gynecological malignancy in developed countries [1]. The 5-year overall survival rate of ovarian cancer patients diagnosed in advanced stages remains lower than 30% despite extensive study for the past decades [4]. EOC makes up more than 90% of ovarian cancer cases and accounts for over 70% of the deaths [10]. In this context, in-depth knowledge about the molecular mechanisms underlying EOC oncogenesis and development may provide new strategies for early diagnosis and treatment of this deadly illness.

BTC is a unique member of the EGF family of ligands. Overexpression of BTC has been observed in multiple types of human cancers [75-77, 79] and up-regulation of BTC is associated with poor outcome in breast cancer [80]. In ovary, several female reproductive processes have been shown to involve BTC expression [219] and expression of BTC mRNA has also been detected in ovarian tumors [220]. However, the specific roles of BTC in ovarian cancer migration, invasion or proliferation, including its signaling, are largely unknown.

Among all the processes modulating ovarian cancer metastasis, EMT is one of the most crucial events. Our previous studies have found that EGF induces ovarian cancer cell migration and invasion by down-regulating E-cadherin expression through a variety of signaling pathways [119-121, 221]. However, if BTC can induce ovarian cancer metastasis by promoting EMT remains unclear.

Gap junctions (Gj) are also very important for different fundamental stages of cancer progression, including cell migration and invasion [222]. Connexin43 (Cx43) is the most extensively studied and widely expressed member of the connexin family [143]. The

physiological roles of Cx43 are diverse however its roles in ovarian cancer progression have not been well studied and remain controversial. Expression of Cx43 was barely detectable in ovarian cancer compared to its moderate level of expression in normal ovarian epithelial cells [175]. EGF has been shown to decrease ovarian cancer cell proliferation by up-regulating Cx43 expression [176]. However, we also found that Cx43 mediates the stimulatory effects of TGF- $\beta$  on ovarian cancer migration [179]. Further studies are required to clarify the exact roles of Cx43 in ovarian cancer metastasis and whether BTC modulates these functions or not.

Besides cell migration and invasion, cell proliferation is also a key step in ovarian cancer progression. CYR61/CCN1 is the first member identified in CCN family of matricellular proteins [200]. CCN1 expression is significantly higher in epithelial ovarian cancer than benign ovarian tumors [209, 212] and overexpression of CCN1 is markedly associated with advanced FIGO stage and poor prognosis [213]. Most importantly, CCN1 has been shown to induce ovarian cancer cell proliferation and inhibit apoptosis via multiple signaling pathways [214-217]. Clarifying the role of BTC in ovarian cancer proliferation and the involvement of CCN1 may provide new molecular targets for ovarian cancer detection and treatment.

## **2.2 Hypothesis**

BTC promotes epithelial ovarian cancer cell migration and proliferation by modulating key cell adhesion, gap junction and matricellular proteins.

## **2.3 Cell models**

SKOV3, OVCAR3, OVCAR4 and OVCAR5 human ovarian cancer cells were obtained from the American Type Culture Collection. Among them, OVCAR4 is well-established as a suitable

model for HGSC: OVCAR4 has a *TP53* mutation but no mutation in non-HGSC genes; its copy-number profile is also highly correlated with the mean copy-number alterations of HGSC tumor samples [223]. OVCAR3 is also suggested to be a HGSC cell line [224]. On the other hand, OVCAR5, which has a *KRAS* mutation but no *TP53* mutation, may not be a HGSC cell line [224]. Note that despite being one of the most commonly used cell lines for studying HGSC, SKOV3 does not closely resemble HGSC because it does not have a *TP53* mutation and instead has *PIK3CA* and *ARID1A* mutations [223]. Previous studies have suggested a clear cell- or endometrioid-like origin of SKOV3 [224].

#### **2.4 Aim of the study**

The general aim of this study was to investigate the roles of BTC in epithelial ovarian cancer migration and proliferation and to investigate potential mechanisms including the involvement of E-cadherin, Cx43 or CCN1 in these processes.

#### **2.5 The specific objectives of this study**

**Objective 1: To investigate the role of BTC in E-cadherin expression and ovarian cancer cell migration.**

- 1) To investigate the effect of BTC on E-cadherin expression and the receptor involvement.
- 2) To determine if transcription factors Snail, Slug or Twist is involved in BTC-reduced E-cadherin expression.
- 3) To examine if common EGF-signaling pathways (MEK/ERK or PI3K/Akt) mediate BTC-induced E-cadherin down-regulation and Slug up-regulation.

- 4) To confirm if BTC induces ovarian cancer cell migration through MEK/ERK and PI3K/Akt signaling pathways via EGFR.

**Objective 2: To investigate the involvement of Cx43 in BTC-induced ovarian cancer cell migration.**

- 1) To investigate the effect of BTC on Cx43 expression in ovarian cancer cells.
- 2) To determine if BTC-induced Cx43 expression is mediated by EGFR.
- 3) To explore the involvement of MEK/ERK and PI3K/Akt signaling pathways in BTC-induced Cx43 expression.
- 4) To investigate the role of Cx43 in BTC-induced ovarian cancer cell migration.

**Objective 3: To investigate the role of BTC in CCN1 expression and ovarian cancer cell proliferation.**

- 1) To investigate the effect of BTC on ovarian cancer cell proliferation.
- 2) To investigate the involvement of EGFR, MEK/ERK and PI3K/Akt in BTC-modulated ovarian cancer cell proliferation.
- 3) To determine if BTC modulates CCN1 expression.
- 4) To explore the involvement of EGFR, MEK/ERK and PI3K/Akt in BTC-regulated CCN1 expression.
- 5) To confirm if CCN1 mediates BTC-induced ovarian cancer cell proliferation.

## **Chapter 3: Betacellulin induces Slug-mediated down-regulation of E-cadherin and cell migration in ovarian cancer cells**

### **3.1 Introduction**

Ovarian cancer is the fifth most common cause of cancer-related death in women and the leading cause of death from gynaecological malignancies [225]. Ovarian cancer patients have a low five-year survival rate (~45%) which is largely attributable to the high proportion of patients presenting with disseminated disease (~60%), for which the survival rate is only ~30% [225, 226]. Epidermal growth factor receptor (EGFR) is overexpressed in a variety of malignancies, including cancers of pancreas, breast, head and neck, lung and ovary [227]. In ovarian cancers, elevated expression of EGFR is correlated with poor prognosis [176, 228-230]. EGFR belongs to the c-erbB receptor tyrosine kinase family, which includes 4 members: EGFR (ERBB1), ERBB2 (HER2), ERBB3 (HER3) and ERBB4 (HER4) [231]. Betacellulin (BTC) is an EGF-like growth factor that binds not only EGFR with high affinity, but also ERBB4 [62]. Overexpression of BTC has been found in many types of human cancers [75-77, 79]. In breast cancer, up-regulation of BTC is associated with reduced disease free survival [80]. In addition, BTC has been shown to act as an autocrine factor promoting the growth of pancreatic tumors [78]. BTC is an important regulator of ovarian follicle development and has been shown to stimulate oocyte maturation and cumulus expansion [219]. BTC mRNA has been detected in ovarian tumors [220], however the functional role and clinical significance of BTC in ovarian cancer remains unknown.

E-cadherin, also known as cadherin 1 (CDH1), is a classical transmembrane cell-cell adhesion glycoprotein and a well-known tumor suppressor. E-cadherin plays an important role in

maintaining normal epithelial cell polarity and structure [95, 232]. Down-regulation of E-cadherin and up-regulation of N-cadherin, often referred to as cadherin switching, is frequently associated with the process of epithelial-mesenchymal transition (EMT). EMTs involve the conversion of polarized, immotile epithelial cells to mesenchymal cells with a motile/invasive phenotype [98, 233]. In ovarian cancer, reduced total or cell surface E-cadherin expression is associated with poor overall or recurrence-free survival [114-116]. In addition, studies have shown that the expression of E-cadherin is negatively correlated with ovarian cancer cell invasiveness [234].

Our previous studies have shown that EGF induces ovarian cancer cell migration and invasion by down-regulating E-cadherin expression through a variety of signaling pathways [119-121, 221]. Whereas BTC may function similar to EGF in many respects, its unique structure and receptor binding properties could result in unique mechanisms and functional roles. Our results show that BTC down-regulates E-cadherin expression and increases cell migration mainly via EGFR in two human ovarian cancer cell lines (SKOV3 and OVCAR5). Although BTC induces the expression of both Snail and Slug, two transcriptional repressors of E-cadherin, only Slug mediates its suppressive effects on E-cadherin expression. Moreover, our results show that both MEK-ERK1/2 and PI3K-Akt signaling pathways are involved in the effects of BTC on E-cadherin and cell migration.

## 3.2 Material and methods

### Cell culture

SKOV3, OVCAR5, OVCAR4 and OVCAR3 human epithelial ovarian cancer cell lines were obtained from American Type Culture Collection. Cells were incubated in a 1:1 (vol/vol) mixture of M199/MCDB105 medium (Sigma-Aldrich) supplemented with 10% (vol/vol) fetal bovine serum (FBS; Hyclone Laboratories) and 1% (vol/vol) penicillin/streptomycin (Gibco). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> and 95% air, and were serum starved for 24 hours prior to treatment.

### Antibodies and reagents

The monoclonal antibodies used in this study were: anti-human E-cadherin (36/E-cadherin, BD Biosciences), anti-human N-cadherin (32/N-cadherin, BD Biosciences), anti-porcine  $\alpha$ -tubulin (B-5-1-2, Santa Cruz Biotechnology), anti-human Snail (L70G2, Cell Signaling Technology), anti-human phospho-ERK1/2 (Thr202/Tyr204) (E10, Cell Signaling Technology), anti-human Slug (C19G7, Cell Signaling Technology), anti-human ERBB2 (29D8, Cell Signaling Technology), anti-human ERBB4 (111B2, Cell Signaling Technology). The polyclonal antibodies used were: anti-rat ERK1/2 (9102, Cell Signaling Technology), anti-human phospho-Akt (9271, Cell Signaling Technology), anti-mouse Akt (9272, Cell Signaling Technology), anti-human EGFR (2232, Cell Signaling Technology). The horseradish peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were obtained from Bio-Rad Laboratories. *E. coli*-derived recombinant human betacellulin (Asp32-Tyr111) was obtained from R&D Systems and diluted in PBS. AG1478 and LY294002 were obtained from Sigma-Aldrich and diluted in

DMSO. U0126 was obtained from Calbiochem and diluted in DMSO. AST1306 (S2185) was obtained from Selleckchem and diluted in DMSO.

### **Small interfering RNA (siRNA) transfection**

To knockdown endogenous Snail or Slug, cells were plated at low density, allowed to recover for 24 hours, and then transfected with 50 nM ON-TARGET<sup>plus</sup>SMARTpool siRNA (Dharmacon) using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. ON-TARGET<sup>plus</sup> non-targeting control pool siRNA (50 nM; Dharmacon) was used as a transfection control in all experiments.

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

Total RNA was extracted using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription was performed with 3 µg of RNA, random primers, and Moloney murine leukemia virus reverse transcriptase (Promega). SYBR Green RT-qPCR was performed on Applied Biosystems 7300 Real-Time PCR System equipped with 96-well optical reaction plates. Each 20 µl RT-qPCR reaction contained 1×SYBR Green PCR Master Mix (Applied Biosystems), 20 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' (sense) and 5'-TCG GAA CCG CTT CCT TCA-3' (antisense); N-cadherin (*CDH2*), 5'-GGA CAG TTC CTG AGG GAT CA-3' (sense) and 5'-GGA TTG CCT TCC ATG TCT GT-3' (antisense); Snail (*SNAI1*), 5'-CCCCAATCGGAAGCCTAACT-3' (sense) and 5'-GCTGGAAGGTAA ACT CTG GAT TAG A-3' (antisense); Slug (*SNAI2*), 5'-TTC GGACCC ACA CAT TAC CT-3' (sense) and 5'-GCAGTGAGGGCAAGA AAA AG-3' (antisense); Twist (*TWIST1*), 5'-GGA GTC CGC AGT

CTT ACG AG-3' (sense) and 5'-TCT GGA GGA CCT GGT AGA GG-3' (antisense); and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), 5'-GAG TCA ACGGAT TTG GTC GT-3' (sense) and 5'-GAC AAG CTT CCC GTTCTC AG-3' (antisense). The amplification parameters were 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. At least three separate experiments were performed on different cultures and each sample was assayed in triplicate. A mean value was used for the determination of mRNA levels by the comparative C<sub>q</sub> method ( $2^{-\Delta\Delta C_q}$ ) with GAPDH as the reference gene.

### **Western blots**

Cells were lysed in lysis buffer (Cell Signaling Technology) containing protease inhibitor cocktail (Sigma-Aldrich). Lysates were centrifuged at 20,000 ×g for 10 minutes at 4°C and supernatant protein concentrations were quantified using the DC Protein Assay (Bio-Rad Laboratories) with BSA (A4503, Sigma-Aldrich) as the standard. 40 µg of protein was loaded in each well. Proteins were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes. After blocking with Tris-buffered saline containing 5% non-fat dry milk for 1 hour, membranes were incubated overnight at 4°C with primary antibodies E-cadherin (1:3000), N-cadherin (1:3000), α-Tubulin (1:3000), phospho-ERK1/2 (1:3000), ERK1/2 (1:3000), phospho-Akt (1:3000), Akt (1:3000), Snail (1:1000), Slug (1:1000), EGFR (1:1000), ERBB2 (1:1000), or ERBB4 (1:1000) followed by incubation with the peroxidase-conjugated secondary antibody (1:5000) for 1 hour at room temperature. Immunoreactive bands were detected with enhanced chemiluminescent or SuperSignal West Femto substrate (Pierce). 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, anti-ERK1/2 or anti-Akt as a loading control.

Immunoreactive band intensities were quantified by densitometry, normalized to those of the relevant loading control, and the results are expressed as fold change relative to the respective control.

### **Transwell migration assays**

Migration assays were performed in Boyden chambers with minor modifications [235]. Transwell cell culture inserts (24-well, pore size 8  $\mu\text{m}$ ; BD Biosciences) were seeded with  $1 \times 10^5$  cells in 250  $\mu\text{L}$  of medium with 0.1% FBS. M199/MCDB15 medium with 10% FBS (750  $\mu\text{L}$ ) was added to the lower chamber and served as a chemotactic agent. After 12 hours incubation, non-migrating cells were wiped from the upper side of the membrane and cells on the lower side were fixed in cold methanol ( $-20^\circ\text{C}$ ) and air dried. Cells were stained with Crystal Violet and counted using a light microscope (10x objective) equipped with a digital camera (QImaging) and Northern Eclipse 6.0 software. Five microscopic fields were counted per insert, triplicate inserts were used for each individual experiment, and each experiment was repeated at least three times.

### **Statistical analysis**

Results are presented as the mean  $\pm$  SEM of at least three independent experiments. PRISM software (GraphPad Software Inc.) was used to perform one-way ANOVA followed by Tukey's multiple comparison test. Means were considered significantly different if  $P < 0.05$  and are indicated by different letters.

### **3.3 Results**

#### **Elevated BTC mRNA expression is associated with reduced disease free survival in ovarian cancer**

To investigate the potential clinical relevance of BTC in ovarian cancer, we queried 489 ovarian cancers from The Cancer Genome Atlas (TCGA [236]) for up-regulation of BTC mRNA above the median. Kaplan-Meier analysis indicates that elevated levels of BTC mRNA are associated with reduced disease free survival (Log-rank  $P=0.0502$ , median 15.54 vs. 18.1 months; Figure 3.1A), albeit not overall survival (Log-rank  $P=0.481$ ; Figure 3.1B). These results suggest that BTC could contribute to poor outcome in ovarian cancer.

#### **Expression levels of ERBB receptors in several epithelial ovarian cancer cell lines**

To evaluate the function of BTC in ovarian cancer cell migration, we first tested the expression levels of EGFR, ERBB2 and ERBB4 in four epithelial ovarian cancer cell lines, SKOV3, OVCAR5, OVCAR4 and OVCAR3. Cells were cultured in M199/MCDB105 medium supplemented with 10% fetal bovine serum for 24 hours and receptor expression levels were examined by Western blot. As shown in Figure 3.2, EGFR and ERBB2 are expressed in all four cell lines to varying degrees. OVCAR5 expressed a higher level of EGFR and SKOV3 expressed an extremely high level of ERBB2 compared to the other cell lines. As for ERBB4, although we did not detect ERBB4 expression in OVCAR5 cells by Western blot, low but detectable mRNA levels of two ERBB4 isoforms were observed by RT-qPCR (data not shown).

#### **BTC down-regulates E-cadherin, but not N-cadherin, via EGFR in ovarian cancer cells**

Next, we examined the effects of BTC on E-cadherin and N-cadherin expression in two ovarian cancer cell lines (SKOV3 and OVCAR5). As shown in Figure 3.3A, treatment for 24 hours with varying concentrations of BTC induced concentration-dependent reductions in E-cadherin mRNA levels in both cell lines, with SKOV3 cells displaying greater sensitivity. In contrast, treatment with BTC did not alter N-cadherin mRNA levels at any of the concentrations tested (Figure 3.3B). Western blot analysis confirmed the suppressive effects of BTC on E-cadherin, but not N-cadherin, protein levels in SKOV3 and OVCAR5 cells (Figure 3.3C). Next, we used the EGFR-specific inhibitor AG1478 to investigate the involvement of EGFR in BTC-induced E-cadherin down-regulation. As shown in Figures 3.3D and 3.3E, pre-treatment of SKOV3 cells with AG1478 completely blocked the down-regulation of E-cadherin mRNA and protein levels by BTC.

### **BTC suppresses E-cadherin via Slug in ovarian cancer cells**

To investigate the involvement of Snail, Slug and/or Twist in BTC-induced E-cadherin down-regulation, we first examined the time-dependent effects of BTC on their mRNA and protein levels in SKOV3 cells. Whereas BTC treatment did not alter Twist mRNA levels (1, 3, 6, 12 or 24 hours; Figure 3.4A), it rapidly induced the mRNA and protein levels of both Snail and Slug, though the increases in Slug were more pronounced and sustained (Figure 3.4A and 3.4B). Consistent with our findings for E-cadherin, these BTC-induced increases in Snail and Slug mRNA and protein levels were abolished by pre-treatment with AG1478 (Figures 3.4C and 3.4D). To further confirm whether Snail or Slug mediates the suppression of E-cadherin by BTC, SKOV3 cells were transfected for 48 hours with Snail or Slug siRNA prior to treatment for 24 hours with BTC. RT-qPCR and Western blot analysis showed that whereas siRNA pre-treatment

specifically down-regulated either Snail or Slug (Figure 3.5A and 3.5B), only knockdown of Slug blocked the suppressive effects of BTC on E-cadherin mRNA (Figure 3.5A) and protein (Figure 3.5B) levels.

### **MEK-ERK and PI3K-Akt signaling contribute to the effects of BTC on E-cadherin and Slug**

To investigate the involvement of MEK-ERK and PI3K-Akt signaling pathways in the effects of BTC on E-cadherin and Slug expression, we first used Western blot to examine their activation following treatment with BTC for 10, 30 or 60 minutes. As shown in Figure 3.6A, treatment with BTC increased the levels of phosphorylated Akt and ERK1/2 at all time-points in both SKOV3 and OVCAR5 cells. In addition, the effects of BTC on Akt and ERK1/2 phosphorylation were abolished by pre-treatment of SKOV3 cells with AG1478 (Figure 3.6B). Next, we used the MEK inhibitor U0126 and the PI3K inhibitor LY294002 to investigate the involvement of these two pathways in BTC-induced down-regulation of E-cadherin and up-regulation of Slug. Pre-treatment of SKOV3 cells with U0126 or LY294002 attenuated the suppressive effects of BTC on E-cadherin mRNA and protein levels (Figure 3.6C). Similarly, the up-regulation of Slug expression by BTC was attenuated by pre-treatment with U0126 or LY294002 (Figure 3.6D).

### **BTC-induced ovarian cancer cell migration requires EGFR, MEK-ERK and PI3K-Akt signaling**

We have previously shown that loss of E-cadherin contributes to EGF-induced ovarian cancer cell invasiveness [120, 237, 238]. To determine whether BTC induces similar pro-migratory

effects, SKOV3 and OVCAR5 cells were treated for 12 hours with BTC and subjected to transwell migration assays. As shown in Figure 3.7A, BTC treatment increased the migration of both SKOV3 and OVCAR5 cells, and this effect was partially abolished by pre-treatment AG1478. In addition, pre-treatment with U0126 or LY294002 reversed the stimulatory effects of BTC on SKOV3 cell migration (Figure 3.7B).

#### **ERBB4 is involved in BTC-induced ovarian cancer cell migration.**

To investigate the involvement of ERBB4 in BTC-induced ovarian cancer cell migration, we used a pan-ERBB inhibitor AST1306, which can effectively inhibit the function of EGFR, ERBB2 and ERBB4 [239]. SKOV3 and OVCAR5 cells were pre-treated for 1 hour with AG1478 or AST1306 prior to BTC treatment, and cell migration was examined by transwell assay. As shown in Figure 3.8, pre-treatment with AST1306 completely abolished BTC-induced SKOV3 and OVCAR5 cell migration.

### **3.4 Discussion**

The fact that most ovarian cancers are diagnosed at advanced stage with widespread peritoneal dissemination is the primary reason for their high mortality, and a persistent therapeutic challenge [225, 240]. EGF-like growth factors have been shown to enhance the invasiveness of ovarian cancer cells by suppressing the expression of E-cadherin [120, 238, 241]. Also a member of this family, BTC has been detected in various human cancers [75-77, 79], where it has been shown to modulate cancer cell growth, invasion and resistance to targeted therapeutics [64, 67, 242]. To date, the potential role of BTC in ovarian cancer remains poorly defined. Previous studies by Tanaka *et al.* failed to show a significant difference in BTC mRNA between normal

ovary and ovarian tumors [220], however most ovarian cancers are thought to arise from the fallopian tube epithelium or the ovarian surface epithelium [243, 244], and comparisons of BTC expression to these cell types have not been reported. Interestingly, however, their results did suggest a trend towards increased BTC expression in stage III-IV tumors [220]. These results are in agreement with our findings that BTC treatment promotes ovarian cancer cell motility, and that BTC is associated with reduced disease free survival. Together with previous studies, our results suggest that enhanced BTC signaling may contribute to ovarian cancer progression, though future studies are required to fully characterize its functional roles and molecular determinants.

BTC has been shown to induce head-and-neck squamous carcinoma cell invasion by up-regulating MMP9 [64, 79]. BTC has also been suggested to induce pancreatic islet migration by modulating RAC1 activity [245]. We now report, for the first time, that BTC down-regulates E-cadherin expression and induces ovarian cancer migration. Besides its putative roles in cancer cell migration/invasion, BTC has also been linked to other processes related to the hallmarks of cancer. For example, several groups have demonstrated the pro-proliferative effects of BTC in pancreatic cancer [78, 246, 247]. Moreover, BTC has been implicated in the development of an inflammatory microenvironment in lung cancer [248]. In addition, BTC has been shown to induce the proliferation and migration of vascular smooth muscle and umbilical vein endothelial cells, indicating a potential role for BTC in angiogenesis [72, 249]. Given that BTC could contribute to poor survival in ovarian cancer, future studies investigating the roles of BTC in ovarian cancer cell invasion, proliferation, apoptosis and angiogenesis would be of interest.

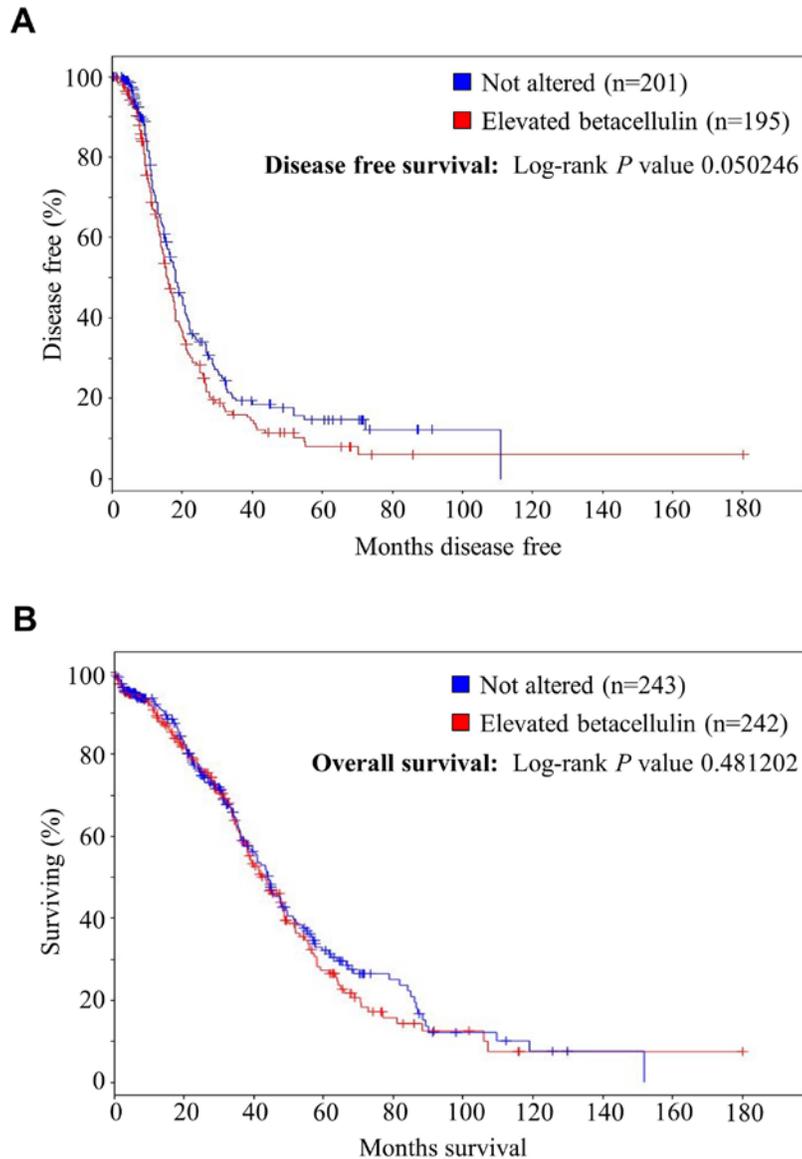
EGF-like growth factors elicit their effects by binding to and activating ERBB receptor homo- or heterodimers [250]. BTC has unique receptor binding properties compared to other well-

studied EGF-like growth factors that bind exclusively to EGFR (e.g. EGF, transforming growth factor- $\alpha$ , amphiregulin). In particular, BTC can bind to either EGFR or ERBB4 and subsequently activate their respective homodimers or all the possible ERBB heterodimers [62]. Previous studies have used AG1478 and an EGFR-specific antagonistic antibody (ICR-62) to demonstrate the importance of EGFR in mediating BTC-induced cell migration and invasion [64, 72]. Similarly, we found that pre-treatment with AG1478 fully blocked BTC-induced E-cadherin down-regulation, Snail and Slug expression, and ERK1/2 and Akt activation. However, BTC-induced SKOV3 and OVCAR5 cell migration was only partially inhibited by AG1478, and totally inhibited by pan-ERBB inhibitor AST1306, suggesting a potential role of ERBB4 in BTC-induced ovarian cancer migration. ERBB4 is the least investigated of all the ERBB family members in ovarian cancer, especially with regards to the effects of BTC. However, several groups have studied the expression and clinical importance of ERBB4 in ovarian tumors [251-254]. Interestingly, mounting evidence suggests that different isoforms of ERBB4 may correlate with different clinical outcomes in ovarian cancer patients [255, 256]. Thus, the role of BTC and ERBB4 in ovarian cancer is likely complex, and warrants further investigation.

Loss of E-cadherin is a key event in epithelial-mesenchymal transition and is associated with poor overall or recurrence-free survival in ovarian cancer [114-116]. We have previously investigated the roles of several E-cadherin transcriptional repressors in mediating the effects of EGF-like growth factors on E-cadherin expression and invasion in ovarian cancer cells [120, 238, 241]. In particular, whereas both Snail and Slug are involved in EGF- and amphiregulin-induced E-cadherin down-regulation, Snail does not participate in the effects of transforming growth factor- $\alpha$  [241]. Interestingly, we show that, like transforming growth factor- $\alpha$ , BTC-induced E-cadherin down-regulation involves Slug, but not Snail. Thus, many of the EGFR-mediated

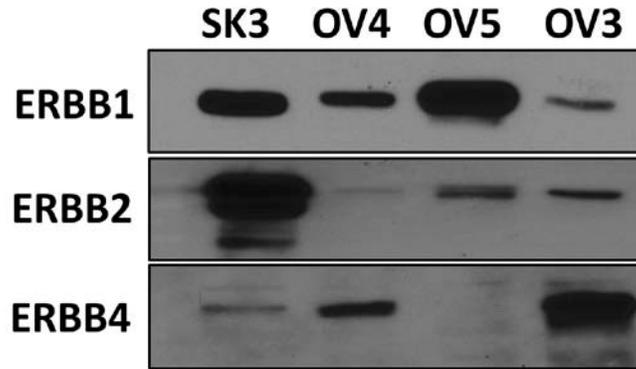
functions of BTC are likely to be similar to other EGF-like growth factors; however BTC could induce a novel subset of effects via ERBB4. In addition to Snail and Slug, we have recently shown that hypoxia-inducible factor-1 $\alpha$ , a key regulator of hypoxic responses [257], also mediates EGF-induced E-cadherin down-regulation and ovarian cancer cell invasion [119]. Interestingly, hypoxia-inducible factor-1 $\alpha$  has been shown to participate in BTC-driven mesenchymal stem cell proliferation [258]. Future studies will be required to examine the effects of BTC on hypoxia-inducible factor-1 $\alpha$  expression in ovarian cancer cells, and whether it may contribute to adaptation to hypoxia, proliferation and/or metastasis.

In summary, our study demonstrates that BTC signals through EGFR to up-regulate Snail and Slug in a MEK-ERK- and PI3K-Akt-dependent manner. Elevation of Slug, not Snail, is required for the down-regulation of E-cadherin expression which promotes ovarian cancer cell migration.



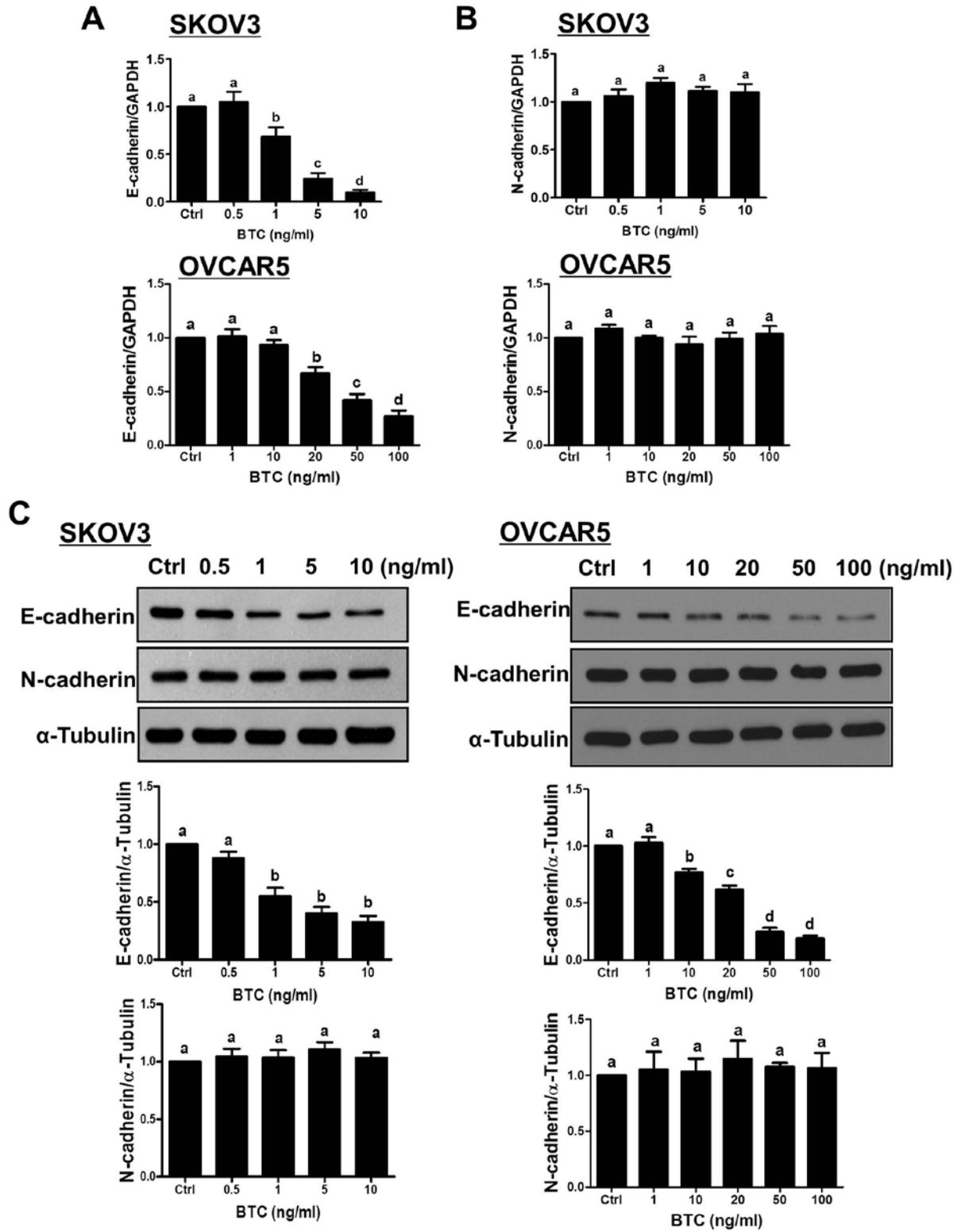
**Figure 3.1. Elevated betacellulin is associated with reduced disease free survival but not overall survival in ovarian cancers.**

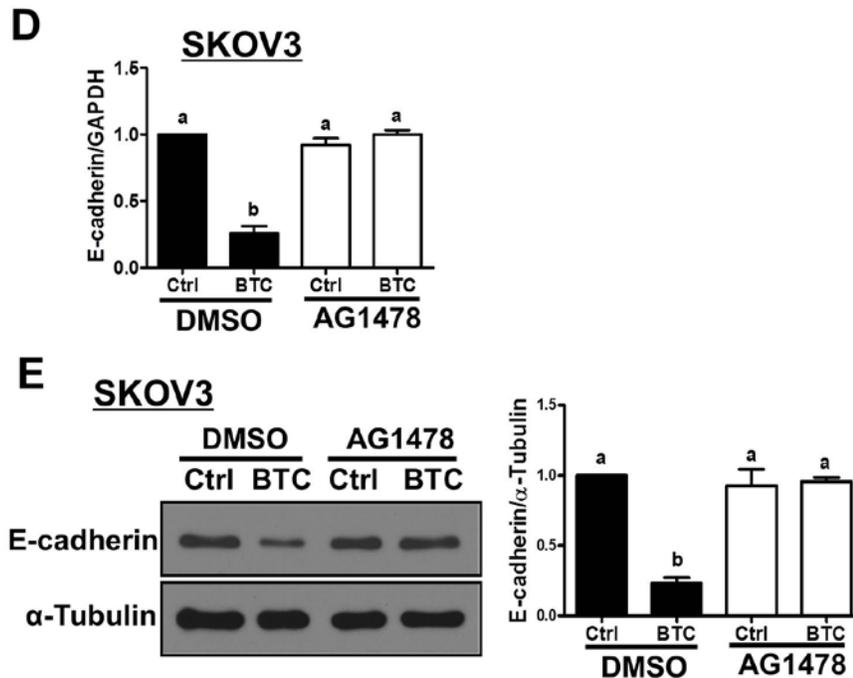
The cBioPortal for Cancer Genomics was used to query ovarian carcinomas from The Cancer Genome Atlas (n=489) for up-regulation of betacellulin mRNA above the median. Disease free (A) and overall (B) survival differences between unaltered samples and those with elevated betacellulin are displayed as Kaplan-Meier survival curves with a  $P$  value from a Log-rank test.



**Figure 3.2. Expression levels of ERBB receptors in several epithelial ovarian cancer cell lines.**

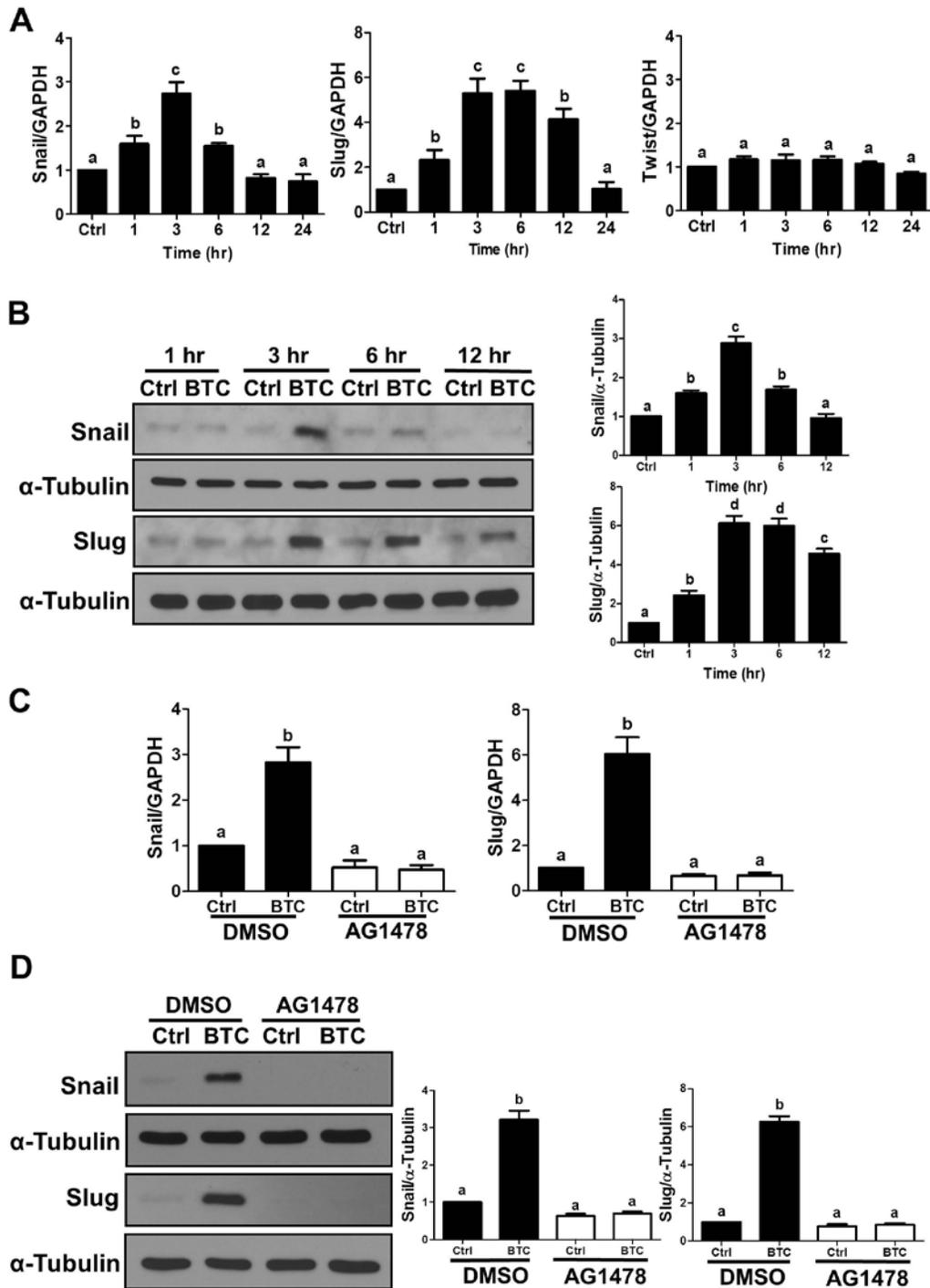
SKOV3, OVCAR4, OVCAR5 and OVCAR3 cells were cultured in M199/MCDB105 medium supplemented with 10% fetal bovine serum for 24 hours, and protein levels of ERBB1, ERBB2 and ERBB4 were measured by Western blot.





**Figure 3.3. Betacellulin down-regulates E-cadherin, but not N-cadherin, via EGFR in ovarian cancer cells.**

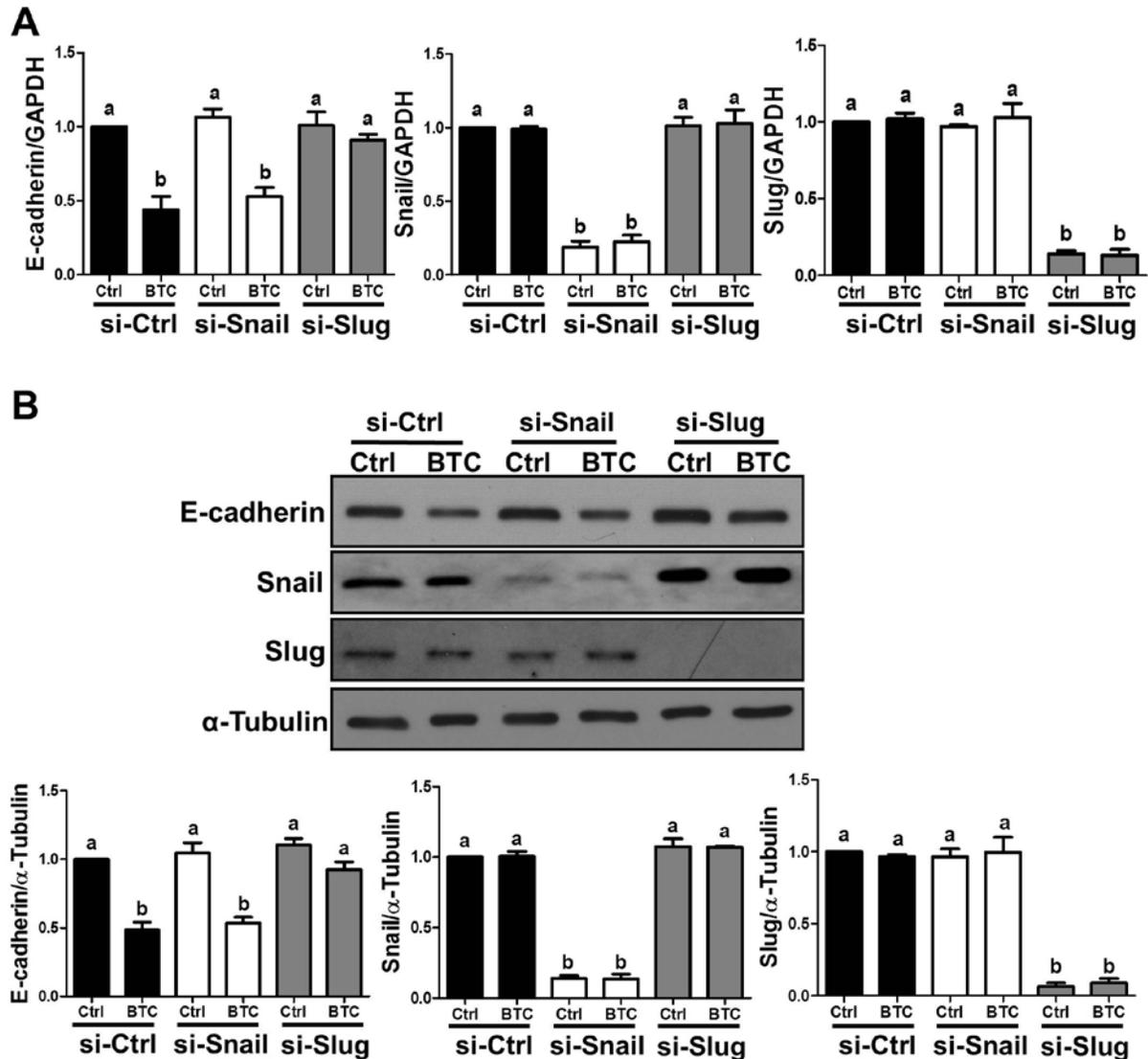
**A-C**, Cells were treated for 24 hours without (Ctrl) or with increasing concentrations of betacellulin (BTC: SKOV3, 0.5, 1, 5 or 10 ng/ml; OVCAR5, 1, 10, 20, 50 or 100 ng/ml), and E-cadherin (**A**) and N-cadherin (**B**) mRNA levels were examined by RT-qPCR. In addition, E-cadherin and N-cadherin protein levels (**C**) were examined by Western blot. **D-E**, SKOV3 cells were pre-treated for 1 hour with vehicle control (DMSO) or 10  $\mu$ M AG1478 prior to treatment with or without 10 ng/ml BTC for 24 hours. E-cadherin mRNA (**D**) and protein (**E**) levels were examined 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 3.4. Betacellulin up-regulates Snail and Slug via EGFR in ovarian cancer cells.**

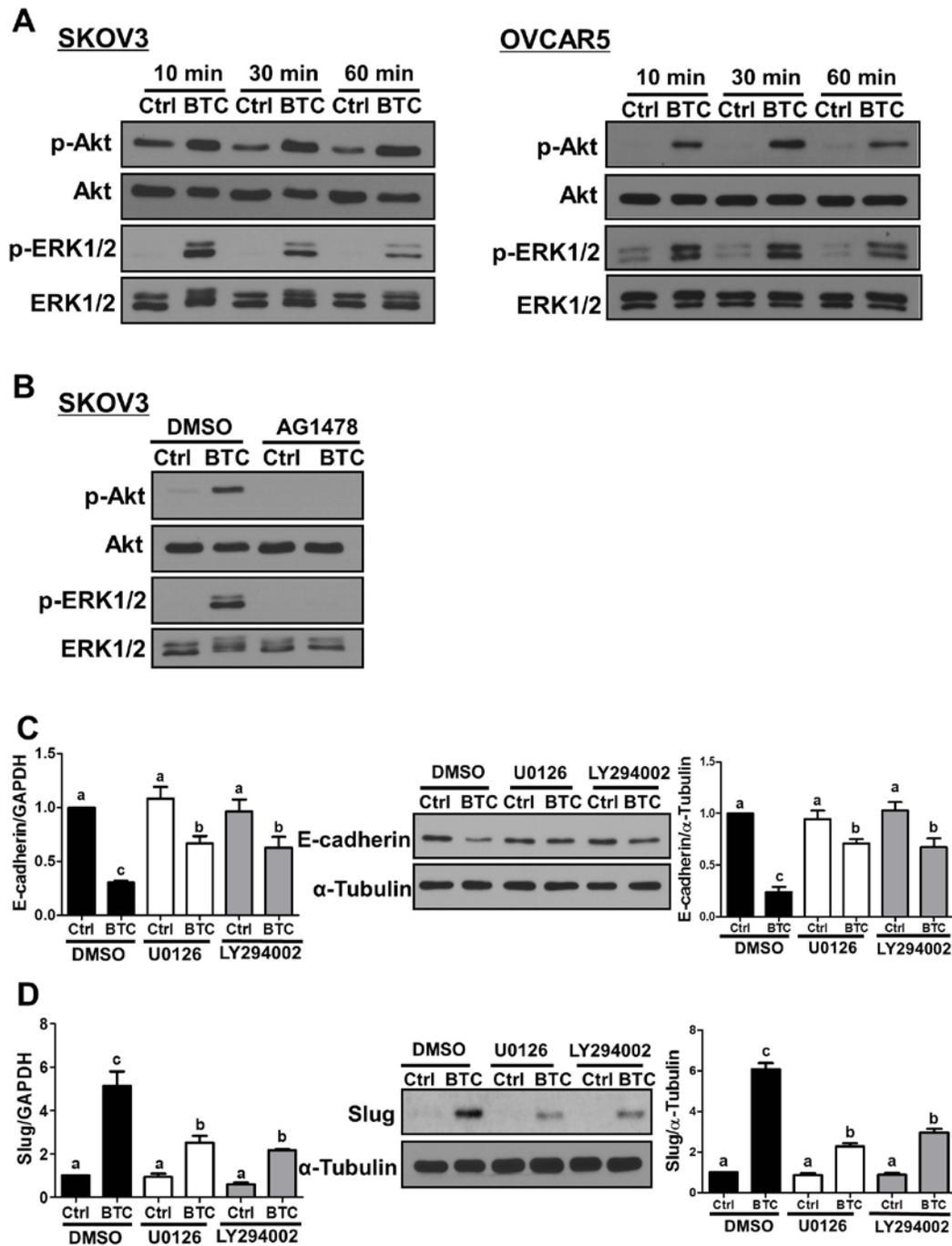
**A-B**, SKOV3 cells were without (Ctrl) or with betacellulin (BTC: 10 ng/ml) for 1, 3, 6, 12 or 24 hours. **A**, Snail, Slug and Twist mRNA levels were examined by RT-qPCR. **B**, Snail and Slug

protein levels were examined by Western blot. **C-D**, SKOV3 cells were pre-treated for 1 hour with vehicle control (DMSO) or 10  $\mu$ M AG1478 prior to treatment with or without 10 ng/ml BTC for 3 hours. Snail and Slug mRNA (**C**) and protein (**D**) levels were examined 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 3.5. Betacellulin suppresses E-cadherin via Slug in ovarian cancer cells.**

SKOV3 cells were transfected for 48 hours with 50 nM non-targeting control siRNA (si-Ctrl) or 50 nM siRNA targeting Snail (si-Snail) or Slug (si-Slug) prior to treatment for 24 hours without (Ctrl) or with 10 ng/ml betacellulin (BTC). E-cadherin, Snail and Slug mRNA (**A**) and protein (**B**) levels were examined 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 ( $P < 0.05$ ).

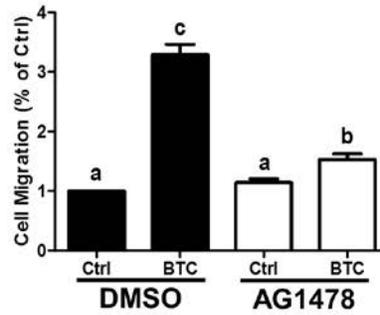
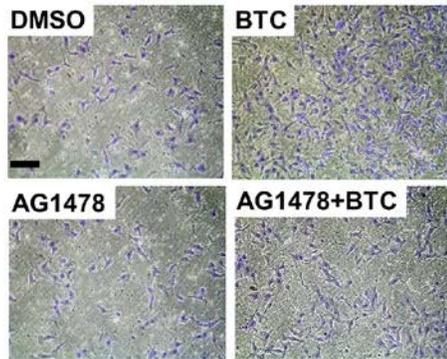


**Figure 3.6. MEK-ERK and PI3K-Akt signaling contribute to the effects of betacellulin on E-cadherin and Slug.**

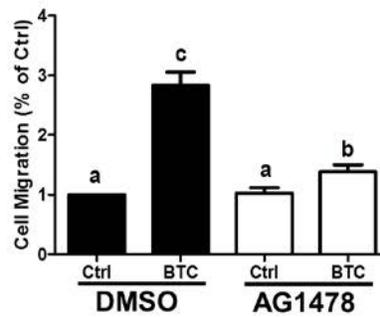
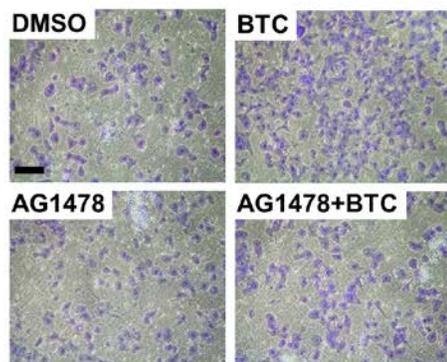
A, Cells were treated without (Ctrl) or with betacellulin (BTC: SKOV3, 10 ng/ml; OVCAR5, 50 ng/ml) for 10, 30 or 60 minutes, and Western blot was used to measure the levels of

phosphorylated Akt (p-Akt) and ERK1/2 (p-ERK1/2) in relation to their total levels (Akt and ERK1/2, respectively). **B**, SKOV3 cells were pre-treated for 1 hour with vehicle control (DMSO) or 10  $\mu$ M AG1478 prior to treatment with or without 10 ng/ml BTC for 30 minutes. Western blot was used to measure the Akt and ERK1/2 phosphorylation/activation. **C-D**, SKOV3 cells were pre-treated for 1 hour with vehicle control (DMSO), 5  $\mu$ M U0126 (MEK inhibitor) or 5  $\mu$ M LY294002 (PI3K inhibitor) prior to treatment with or without 10 ng/ml BTC. E-cadherin (24 hours; **C**) and Slug (3 hours; **D**) mRNA and protein levels were examined by RT-qPCR and Western blot. Results are expressed as the mean  $\pm$  SEM of at least three independent experiments and values without common letters are significantly different ( $P < 0.05$ ).

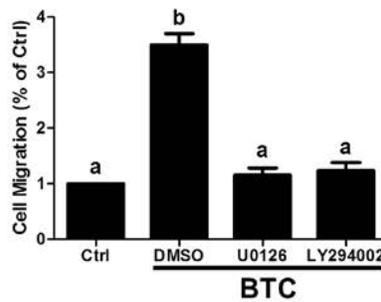
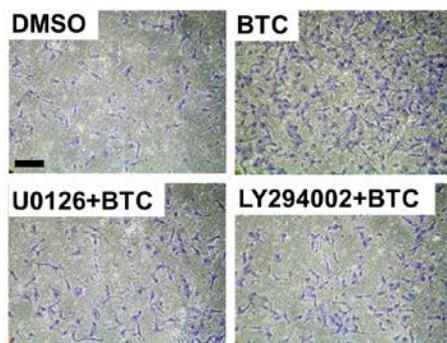
### A SKOV3



### OVCAR5



### B SKOV3

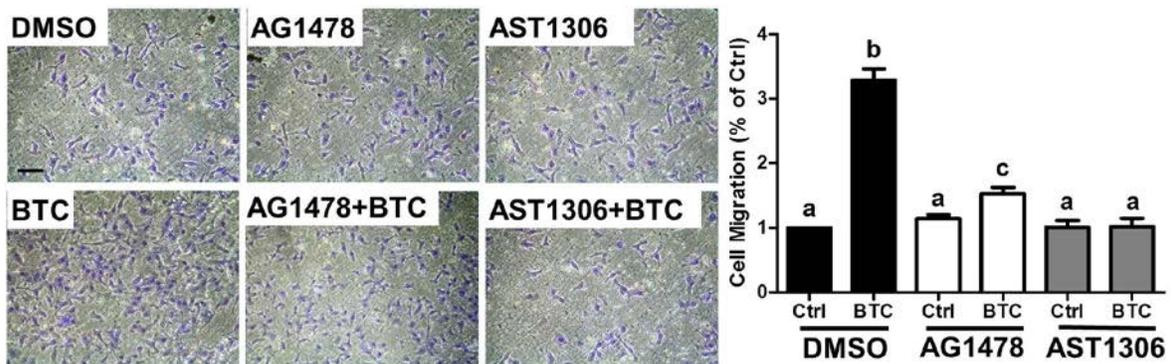


**Figure 3.7. Betacellulin-induced ovarian cancer cell migration requires EGFR, MEK-ERK and PI3K-Akt signaling.**

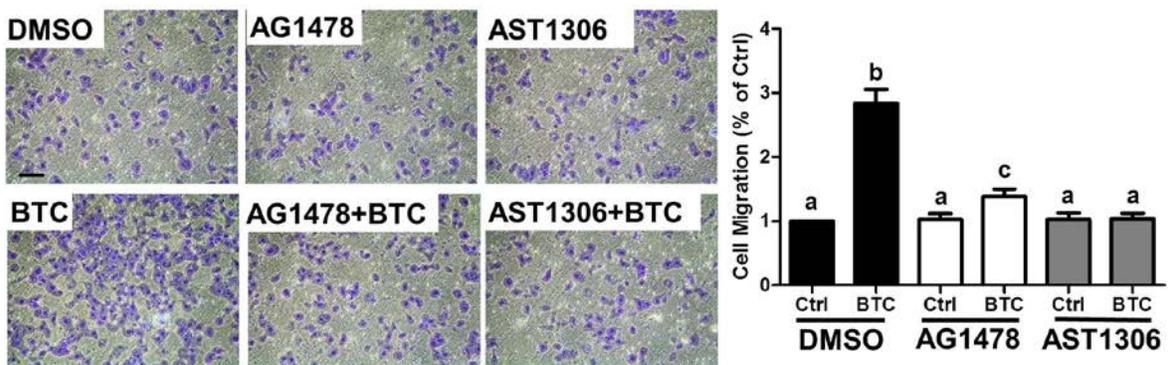
A, SKOV3 and OVCAR5 cells were pre-treated for 1 hour with vehicle control (DMSO) or 10  $\mu$ M AG1478 prior to treatment without (Ctrl) or with betacellulin (BTC: 10 ng/ml for SKOV3,

50 ng/ml for OVCAR5), and cell migration was examined by transwell assay (12 hours). **B**, SKOV3 cells were pre-treated for 1 hour with or without 5  $\mu$ M U0126 or 5  $\mu$ M LY294002 prior to treatment with 10 ng/ml BTC, and cell migration was examined by transwell assay (12 hours). Cells were stained with Crystal Violet; and pictures were taken under light microscope (10x objective) equipped with a digital camera. Results are expressed as the mean  $\pm$  SEM of at least three independent experiments and values without common letters are significantly different ( $P < 0.05$ ).

### SKOV3



### OVCAR5



**Figure 3.8. ERBB4 is involved in BTC-induced ovarian cancer cell migration.**

SKOV3 and OVCAR5 were pre-treated for 1 hour with vehicle control (DMSO), 10  $\mu$ M AG1478 (EGFR inhibitor) or 1  $\mu$ M AST1306 (Pan-ERBB inhibitor) prior to treatment without (Ctrl) or with betacellulin (BTC: 10 ng/ml for SKOV3, 50 ng/ml for OVCAR5), and cell migration was examined by transwell assay (12 hours). Results are expressed as the mean  $\pm$  SEM of at least three independent experiments and values without common letters are significantly different ( $P < 0.05$ ).

## **Chapter 4: Betacellulin enhances ovarian cancer cell migration by up-regulating Connexin43 via MEK-ERK signaling**

### **4.1 Introduction**

Ovarian cancer is the fifth leading cause of cancer-related death in women in developed countries [1]. In 2016, there were an estimated 22,280 new ovarian cancer cases and 14,230 deaths in United States, which is the highest among all the gynaecological malignancies [1]. Despite the progression in conventional therapies such as surgery and chemotherapy in the past decades, the 5-year survival of ovarian cancer remains lower than 50%. The main reasons for this situation are lack of accurate screening test for early diagnosis and the high metastatic ability of ovarian cancer cells. The 5-year survival of ovarian cancer patients diagnosed at late stage is only 28%, compared to the 92% when diagnosed at early stages when the disease is localized [1].

Gap junctions are specialized channel structures between adjacent cells [126]. They are essential for numerous physiological activities such as embryonic development, cell proliferation and differentiation, and are also involved in many pathological processes [125, 131]. Gap junctions are formed by connexin (Cx) subunit proteins. Among all the connexin family members, connexin43 (Cx43) is the most studied and widely expressed gap junction protein [143, 144]. Cx43 was initially considered to be a tumor-suppressor because reduced expression of Cx43 has been observed in various types of cancer [168]. The expression of Cx43 was barely detectable in ovarian tumors compared to moderate expression in normal ovarian epithelial cells [175]. Our previous study also showed that epidermal growth factor (EGF)-induced Cx43 expression negatively regulated ovarian cancer cell proliferation [176]. However, increasing

evidence suggests the involvement of Cx43 in oncogenesis and cancer progression [177, 178, 259]. Elevated Cx43 mRNA expression was shown to be associated with reduced overall survival in the TCGA database of 489 high-grade serous ovarian cancer cases [176], and we have also found that Cx43 mediates the stimulatory effects of TGF- $\beta$  on ovarian cancer cell migration [179, 260]. Taken together, the roles of Cx43 in ovarian cancer development and progression may be more complex than previously thought and are worth further investigating.

Betacellulin (BTC) is a ligand for the ERBB receptor family, which contains 4 members: ERBB1 (EGFR), ERBB2 (HER2), ERBB3 (HER3) and ERBB4 (HER4). BTC can bind to both ERBB1 and ERBB4, and also activates all possible heterodimeric combinations of ERBB receptors [261]. These binding properties give BTC unique biological functions that are distinct from many of the ligands in this family. Overexpression of BTC has been detected in several types of human malignancies [76, 77], and up-regulation of BTC was shown to be associated with poor clinical outcome in breast cancer [80]. Our previous study demonstrated that BTC treatment induced ovarian cancer cell migration, likely by down-regulating E-cadherin [262]. However, the specific functions of BTC in ovarian cancer progression and their underlying mechanisms are still largely unknown.

Given the importance of Cx43 in ovarian cancer progression and known links between Cx43 and EGF signaling [176], we hypothesize that Cx43 can be increased by BTC and is positively involved in BTC-induced ovarian cancer cell migration. Our results show that BTC up-regulates Cx43 expression and cell migration in two ovarian cancer cell lines (OVCAR4 and SKOV3), and these effects are primarily mediated by MEK-ERK signaling via activation of EGFR. Cx43 is involved in BTC-induced ovarian cancer cell migration however its effects are likely mediated in a gap junction-independent manner.

## 4.2 Material and methods

### Cell culture

OVCAR4 and SKOV3 human epithelial ovarian cancer cell lines were obtained from American Type Culture Collection. Cells were incubated in a 1:1 (vol/vol) mixture of M199/MCDB105 medium (Sigma-Aldrich) supplemented with 10% (vol/vol) fetal bovine serum (FBS; Hyclone Laboratories) and 1% (vol/vol) penicillin/streptomycin (Gibco). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> and 95% air, and were serum starved for 24 hours prior to treatment.

### Antibodies and reagents

The monoclonal antibodies used in this study were: anti-porcine  $\alpha$ -tubulin (B-5-1-2, Santa Cruz Biotechnology), anti-human phospho-ERK1/2 (Thr202/Tyr204) (E10, Cell Signaling Technology). The polyclonal antibodies used were: anti-human Cx43 (3512, Cell Signaling Technology), anti-rat ERK1/2 (9102, Cell Signaling Technology), anti-human phospho-Akt (9271, Cell Signaling Technology), anti-mouse Akt (9272, Cell Signaling Technology). The horseradish peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were obtained from Bio-Rad Laboratories. *E. coli*-derived recombinant human betacellulin (Asp32-Tyr111) was obtained from R&D Systems and diluted in PBS. *E. coli*-derived recombinant human neuregulin-4 was obtained from Thermo Fisher Scientific and diluted in PBS. Human recombinant epidermal growth factor (E9644) was obtained from Sigma-Aldrich and diluted in PBS. AG1478, LY294002 and carbenoxolone were obtained from Sigma-Aldrich and diluted in DMSO. U0126 was obtained from Calbiochem and diluted in DMSO.

### **Small interfering RNA (siRNA) transfection**

To knock down endogenous Cx43, cells were plated at low density, allowed to recover for 24 hours, and then transfected with 50 nM ON-TARGET*plus*SMARTpool siRNA (Dharmacon) using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. ON-TARGET*plus* non-targeting control pool siRNA (50 nM; Dharmacon) was used as a transfection control in all experiments.

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

Total RNA was extracted using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription was performed with 3 µg of RNA, random primers, and Moloney murine leukemia virus reverse transcriptase (Promega). SYBR Green RT-qPCR was performed on Applied Biosystems 7300 Real-Time PCR System equipped with 96-well optical reaction plates. Each 20 µl RT-qPCR reaction contained 1×SYBR Green PCR Master Mix (Applied Biosystems), 20 ng cDNA and 150 nM of each specific primer. The primers used were: Cx43, 5'-TAC CAA ACA GCA GCGGAG TT-3' (sense) and 5'-TGG GCA CCA CTC TTT TGC TT-3'(antisense); and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), 5'-GAG TCA ACGGAT TTG GTC GT-3' (sense) and 5'-GAC AAG CTT CCC GTTCTC AG-3' (antisense). The amplification parameters were 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. At least three separate experiments were performed on different cultures and each sample was assayed in triplicate. A mean value was used for the determination of mRNA levels by the comparative C<sub>q</sub> method ( $2^{-\Delta\Delta C_q}$ ) with *GAPDH* as the reference gene.

## **Western blots**

Cells were lysed in lysis buffer (Cell Signaling Technology) containing protease inhibitor cocktail (Sigma-Aldrich). Lysates were centrifuged at 20,000 ×g for 10 minutes at 4°C and supernatant protein concentrations were quantified using the DC Protein Assay (Bio-Rad Laboratories) with BSA (A4503, Sigma-Aldrich) as the standard. 40 µg of protein was loaded in each well. Proteins were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes. After blocking with Tris-buffered saline containing 5% non-fat dry milk for 1 hour, membranes were incubated overnight at 4°C with primary antibodies Cx43 (1:1000),  $\alpha$ -Tubulin (1:3000), phospho-ERK1/2 (1:3000), ERK1/2 (1:3000), phospho-Akt (1:3000) or Akt (1:3000), followed by incubation with the peroxidase-conjugated secondary antibody (1:5000) for 1 hour at room temperature. Immunoreactive bands were detected with enhanced chemiluminescent or SuperSignal West Femto substrate (Pierce). Membranes were stripped with stripping buffer (50 mM Tris-HCl pH 7.6, 10 mM  $\beta$ -mercaptoethanol and 1% SDS) at 50°C for 30 minutes and reprobed with anti- $\alpha$ -Tubulin, anti-ERK1/2 or anti-Akt as a loading control. Immunoreactive band intensities were quantified by densitometry, normalized to those of the relevant loading control, and the results are expressed as fold change relative to the respective control.

## **Transwell migration assays**

Migration assays were performed in Boyden chambers with minor modifications [235]. Transwell cell culture inserts (24-well, pore size 8 µm; BD Biosciences) were seeded with  $1 \times 10^5$  cells in 250 µL of medium with 0.1% FBS. Medium with 10% FBS (750 µl) was added to the lower chamber and served as a chemotactic agent. After 24 hours incubation, non-migrating cells were wiped from the upper side of the membrane and cells on the lower side were fixed in cold

methanol (-20°C) and air dried. Cells were stained with Crystal Violet and counted using a light microscope (10x objective) equipped with a digital camera (QImaging) and Northern Eclipse 6.0 software. Five microscopic fields were counted per insert, triplicate inserts were used for each individual experiment, and each experiment was repeated at least three times.

### **Statistical analysis**

Results are presented as the mean  $\pm$  SEM of at least three independent experiments. PRISM software (GraphPad Software Inc.) was used to perform one-way ANOVA followed by Tukey's multiple comparison test. Means were considered significantly different if  $P < 0.05$  and are indicated by different letters.

## **4.3 Results**

### **BTC induces Cx43 expression in ovarian cancer cells**

To investigate whether BTC regulates Cx43 expression, we first treated two ovarian cancer cell lines (OVCAR4 and SKOV3) with BTC (100 ng/ml) for different amounts of time (1-24 hours). As shown in Figure 4.1A-B, BTC treatment up-regulated Cx43 mRNA and protein levels in a time-dependent manner in both cell lines, with maximum effects at 24 hours. Next, cells were treated with varying concentrations of BTC for 24 hours and the RT-qPCR results showed that BTC induced Cx43 expression in a concentration-dependent manner, with more than a 3-fold increase at a concentration of 50ng/ml (Figure 4.1C). Similar up-regulation of Cx43 protein levels was confirmed by Western blot following BTC treatment for 24 hours (Figure 4.1D).

### **BTC up-regulates Cx43 partially via EGFR**

To investigate the receptor requirements of BTC-induced Cx43 production, we pre-treated OVCAR4 and SKOV3 cells with the EGFR-specific inhibitor AG1478 for 1 hour prior to BTC treatment for 24 hours. As shown in Figure 4.2A, pre-treatment with AG1478 partially attenuated the up-regulation of Cx43 mRNA by BTC in both cell lines. Western blot results showed a similar suppressive effect of AG1478 on BTC-induced Cx43 protein levels (Figure 4.2B).

### **MEK-ERK signaling mediates BTC-induced Cx43 expression**

To investigate the signaling pathways involved in BTC-induced Cx43 expression, we first treated SKOV3 and OVCAR4 cells with BTC (50 ng/ml) for 10, 30 or 60 minutes and used Western blot to measure the phosphorylation/activation of ERK1/2 and Akt, two common signaling mediators for EGF-like growth factors. As shown in Figure 4.3A, the phosphorylation of both ERK1/2 and Akt was increased at all time-points in BTC-treated SKOV3 cells. Likewise, 30 min BTC treatment increased the levels of phosphorylated ERK1/2 and Akt in OVCAR4 cells, although the activation of Akt was much lower than that of EGF-treated positive control cells. In addition, pre-treatment with AG1478 totally blocked the stimulatory effects of BTC on ERK1/2 and Akt phosphorylation in both cell lines (Figure 4.3B).

Next, we used the MEK inhibitor U0126 and the PI3K inhibitor LY294002 to investigate the involvement of these two pathways in BTC-induced Cx43 expression. Pre-treatment of OVCAR4 and SKOV3 cells with U0126 blocked the up-regulation of Cx43 mRNA and protein levels by BTC. In contrast, pre-treatment with LY294002 only partially attenuated the effects of

BTC in SKOV3 cells whereas it did not alter the effects of BTC in OVCAR4 cells (Figure 4.3C and 4.3D).

### **Cx43 is involved in BTC-induced ovarian cancer cell migration**

We have previously shown that treatment with BTC enhances SKOV3 and OVCAR5 ovarian cancer cell migration (12 hours) [262]. To confirm this effect in our current cell models, BTC-treated OVCAR4 and SKOV3 cells were cultured in transwell migration assays for 24 hours. As shown in Figure 4.4A, treatment with BTC (50 ng/ml) enhanced the migration of both cell lines, and these effects were partially abolished by AG1478 pre-treatment.

Previous studies have shown that knockdown of Cx43 attenuates TGF- $\beta$ -stimulated OVCAR4 and SKOV3 cell migration [179]. To determine whether Cx43 is required for BTC-induced cell migration, we transfected OVCAR4 and SKOV3 cells with Cx43 siRNA for 48 hours prior to BTC-treatment and subsequent analysis of transwell migration. Pre-treatment with Cx43 siRNA significantly reduced Cx43 protein levels and partially reversed BTC-induced transwell cell migration (Figure 4.4B). EGF was used as a positive control and knockdown of Cx43 had similar inhibitory effects on migration induced by this ligand (Figure 4.4B).

### **Neuregulin-4 induces SKOV3 cell migration via Cx43**

As shown in Figure 4.2A, pre-treatment with AG1478 only partially attenuated BTC-induced Cx43 expression, which suggested the potential involvement of ERBB4. To further investigate the role of ERBB4 in BTC-induced ovarian cancer cell migration and if Cx43 is involved in ERBB4 signaling, we used neuregulin-4 (NRG4), an EGF-like ligand that binds exclusively with ERBB4 [263]. SKOV3 cells were transfected with Cx43 siRNA for 48 hours prior to NRG4

treatment and subsequent analysis of transwell migration. As shown in Figure 4.5, NRG4 significantly increased SKOV3 cell migration and knockdown of endogenous Cx43 partially attenuated this effect.

### **BTC induces ovarian cancer cell migration in a gap junction-independent manner**

Cx43 has been shown to modulate cell migration via both gap junction-dependent and -independent mechanisms [264]. To investigate if gap junction intercellular communication is required for the effects of BTC on OVCAR4 and SKOV3 cell migration, we examined its effects following pre-treatment with or without Cx43 siRNA in the presence or absence of the well-known gap junction inhibitor carbenoxolone [265]. Western blot results showed that carbenoxolone treatment had no effect on Cx43 protein levels (Figure 4.6A). In control siRNA-treated cells, pre-treatment with carbenoxolone did not alter the stimulatory effects of BTC on OVCAR4 or SKOV3 cell migration (Figure 4.6B). Similarly, although Cx43 siRNA treatment reduced BTC-induced cell migration, combined treatment with carbenoxolone did not result in further reduction in OVCAR4 or SKOV3 cell migration (Figure 4.6B).

## **4.4 Discussion**

Previous studies have shown that overexpression of Cx43 attenuated EGF-induced ovarian cancer cell proliferation [176]. However, such a finding appears to conflict with the negative correlation between Cx43 mRNA expression and overall survival of ovarian cancer patients in the TCGA dataset [176]. In the current study, we found that Cx43 positively regulated BTC-induced ovarian cancer cell migration, which may be indicative of cellular functions that could explain the above mentioned correlation. Peritoneal implantation of ovarian cancer cells in later-

stages is one of the reasons why ovarian cancer patients have high mortality. Five-year survival rates of patients who have disseminated disease is only 28% compared to 92% for patients whose tumor are still localized [1]. Considering this, the high motility and metastatic capability of ovarian cancer cells could be a vital factor for cancer development and prognosis. Our results suggest that the roles of Cx43 in ovarian cancer progression are multifaceted and Cx43 may act to promote ovarian cancer progression or metastasis by enhancing cell migration. However, this effect needs to be further confirmed and the roles of Cx43 in other cell functions related to the metastatic process (e.g. invasion and adhesion) require further investigation.

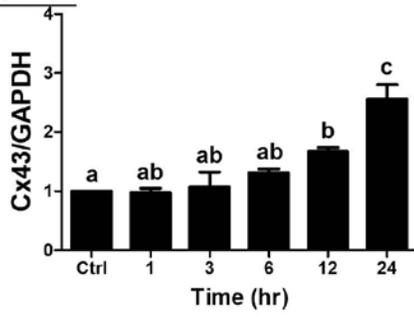
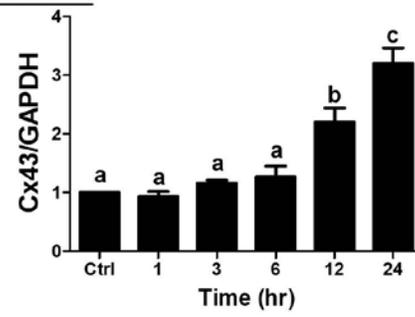
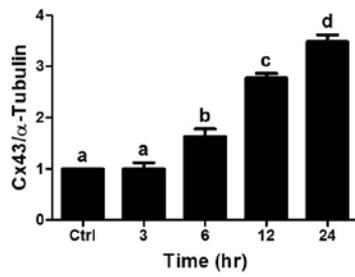
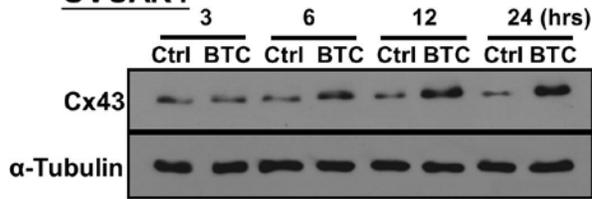
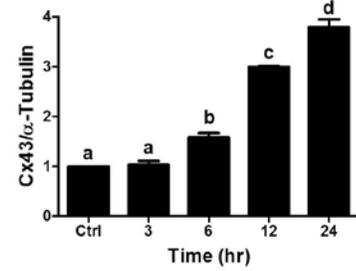
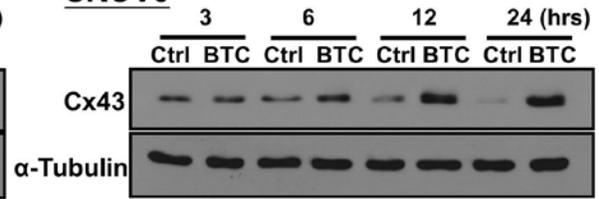
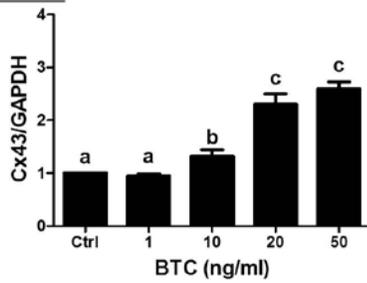
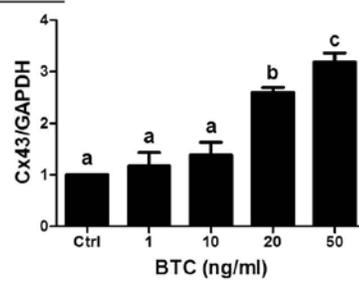
As a member of the EGF-like growth factor family, BTC works similarly to EGF in many respects. However, BTC's ability to bind with ERBB4 as well as EGFR may give BTC unique functions. In the present study, the EGFR-specific inhibitor AG1478 only partially attenuated BTC-induced Cx43 expression and cell migration, and NRG4 induced ovarian cancer cell migration, suggesting a potential role for ERBB4. Indeed, our previous study showed that pre-treatment with the pan-ERBB inhibitor AST1306 totally blocks BTC-induced SKOV3 cell migration (Figure 3.8). Several research groups have observed a high incidence of ERBB4 expression in ovarian cancer [251, 254, 255]; and ERBB4 has also been shown to correlate with serous histological subtype and cisplatin resistance [255]. However, no specific study has investigated how ERBB4 contributes to the functions of BTC in cancer or its potential mechanisms. Future studies will be required to further confirm the contributions of ERBB4 signaling to BTC-regulated cellular functions in ovarian cancer, and to clarify the signaling pathways involved.

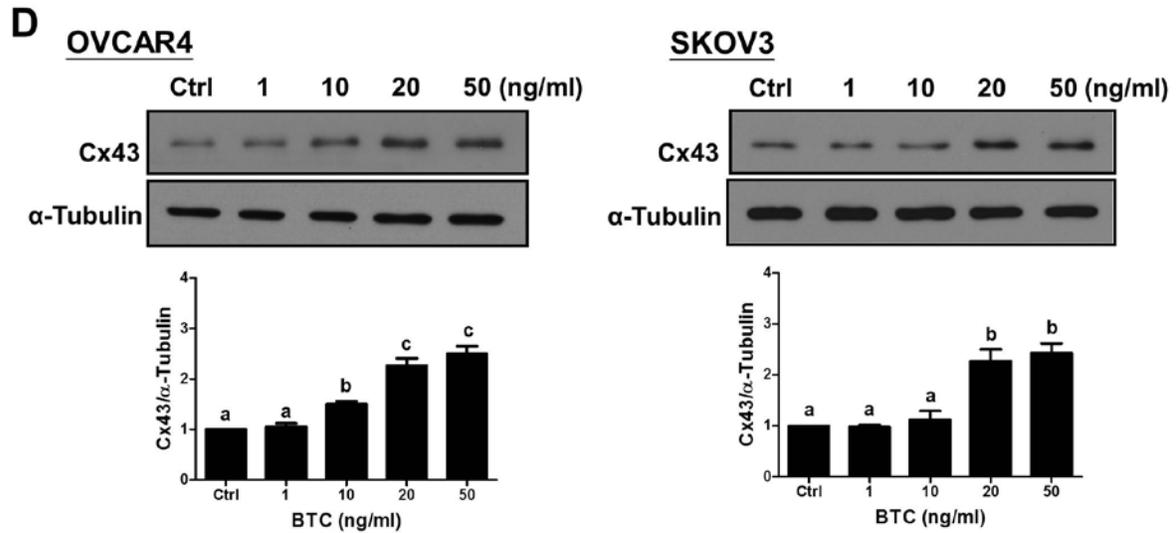
Gap junctional intercellular communication (GJIC) is a double-edge sword in tumorigenesis. Gap junctions were initially implicated in tumor suppression since they played crucial roles in

suppressing cell proliferation and maintaining tissue homeostasis. However, increasing evidence has implicated GJIC in cell adhesion, migration and invasion, mostly in a manner that would promote cancer development and progression [264, 266, 267]. Carbenoxolone is an effective agent to block GJIC and its efficiency in our cell line models SKOV3 and OVCAR4 has been previously confirmed using the scrape-loading dye transfer assay [176]. In our study, pre-treatment with carbenoxolone altered neither BTC-induced ovarian cancer cell migration nor the partial inhibition of BTC-induced cell migration by Cx43 siRNA, suggesting that the contributions of Cx43 to BTC-induced ovarian cancer migration are independent of GJIC. The gap junction-independent roles of connexins have been increasingly studied in the past decades. One of the mechanisms by which Cx43 enhances cell migration independent of channel function is the extracellular adhesive guidance of hemichannels [264]. A systematic study has shown that expression of Cx43 hemichannels induced adhesive aggregation between C6-glioma and HeLa cells, which were commonly incompatible to each other [268]. Several studies also suggested that the migration pattern of interneurons depend on Cx43 adhesion but not Cx43 channel formation [269, 270]. Besides the adhesive effect conducted by Cx43 extracellular domain, the carboxyl tail of Cx43 can extensively interact with multiple structural proteins, signaling molecules or enzymes, and modulate cell migration [267]. By interacting with cytoskeletal proteins such as microtubules and actin, Cx43 is able to control cell polarity and the formation of protrusive structures [264]. Cx43 can also interact with tight junctional protein ZO-1 and adherens junctional protein N-cadherin and E-cadherin, modulating cell adhesion and further influence migration [264]. Notably, one study showed that E-cadherin could modulate the transportation of Cx43 from the cytoplasm to points of cell-cell contact [271]; and as I presented in Chapter 3, BTC-induced ovarian cancer migration involves the down-regulation of E-cadherin

[262]. Lack of E-cadherin and adherens junction-mediated cell-cell adhesion may also emphasize the GJIC-independent role of Cx43. Besides these junctional proteins, Cx43 can also interact with  $\beta$ -catenin and may therefore be involved in modulating the transcription of genes that control cell migration [154]. Furthermore, the phosphorylation of Cx43 carboxyl tail by protein kinases such as Src and ERK can alter hemichannel permeability and assembly, and further affect migration [272]. In the current study, we only showed that BTC up-regulates Cx43 expression and promotes cell migration. However, whether the pro-migratory effects of BTC are driven by up-regulation of Cx43 *vs.* enhanced modification of Cx43 (e.g. phosphorylation) is still unclear. Taken together, the GJIC-independent roles of Cx43 are sophisticated; future studies will be required to clarify the precise mechanisms by which Cx43 contributes to BTC-induced ovarian cancer cell migration.

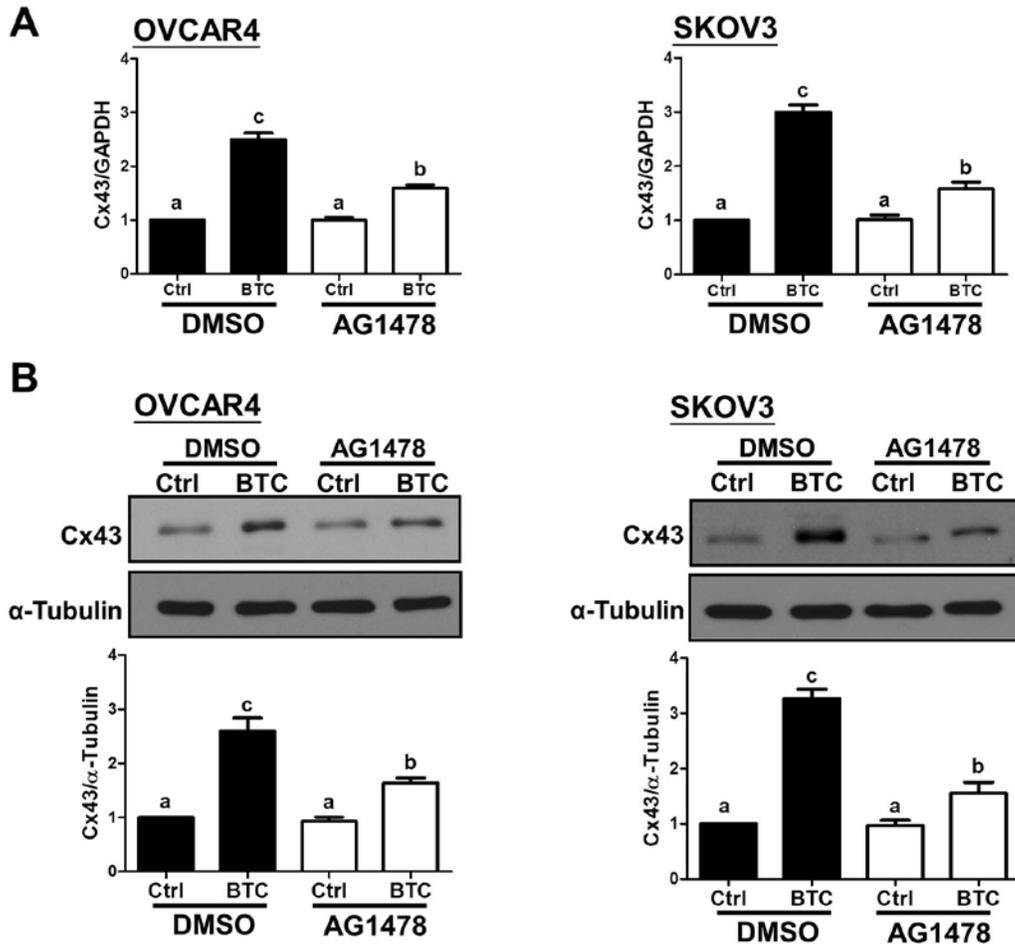
In summary, our study suggests that Cx43 may promote ovarian cancer progression and further elucidates how BTC enhances ovarian cancer migration. We show that BTC induces Cx43 expression and ovarian cancer cell migration mainly through EGFR via MEK/ERK signaling. Furthermore, Cx43 is required for BTC-induced cell migration however this effect does not appear to involve channel function.

**A** OVCAR4**SKOV3****B** OVCAR4**SKOV3****C** OVCAR4**SKOV3**



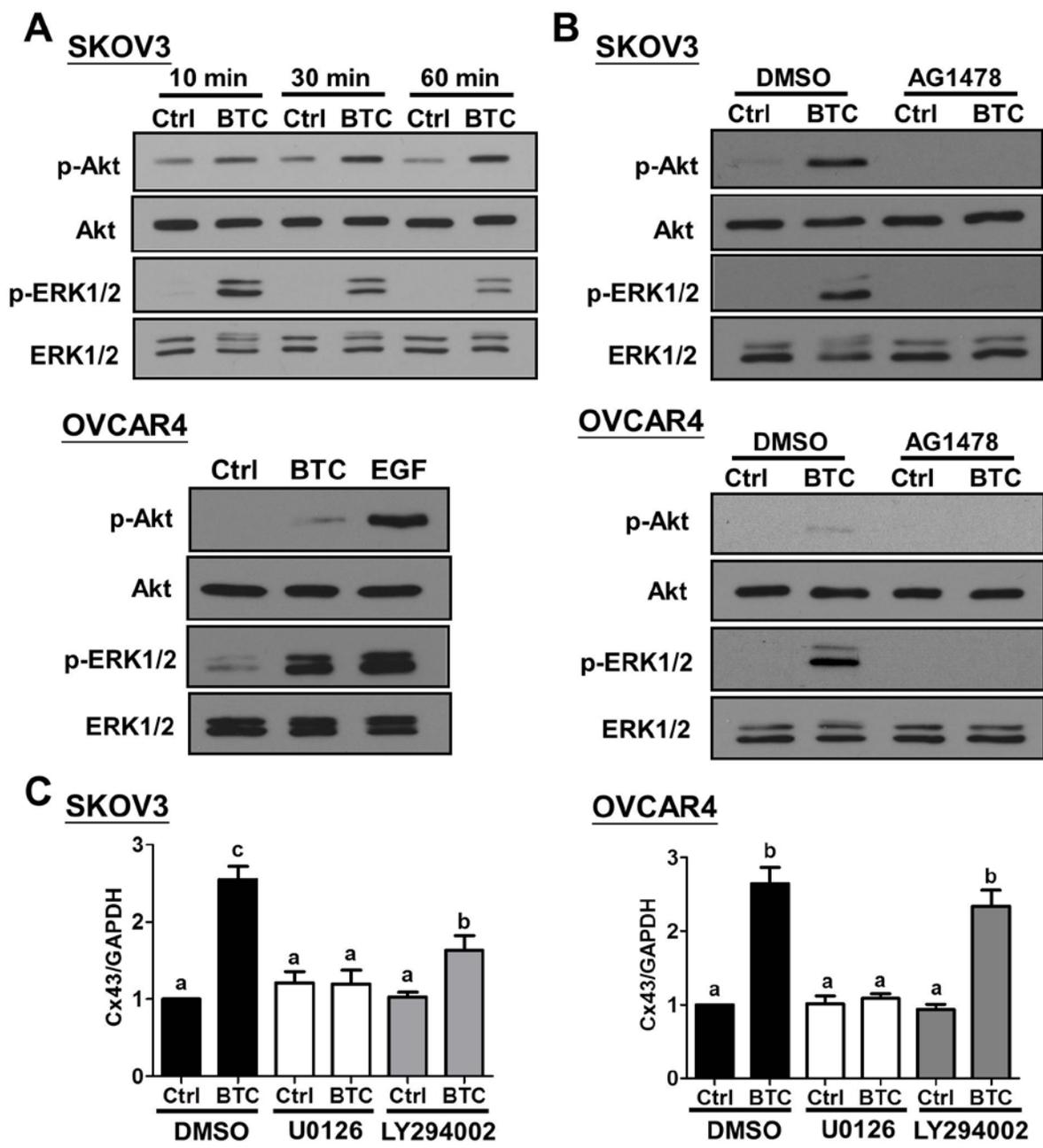
**Figure 4.1. BTC induces Cx43 expression in ovarian cancer cells**

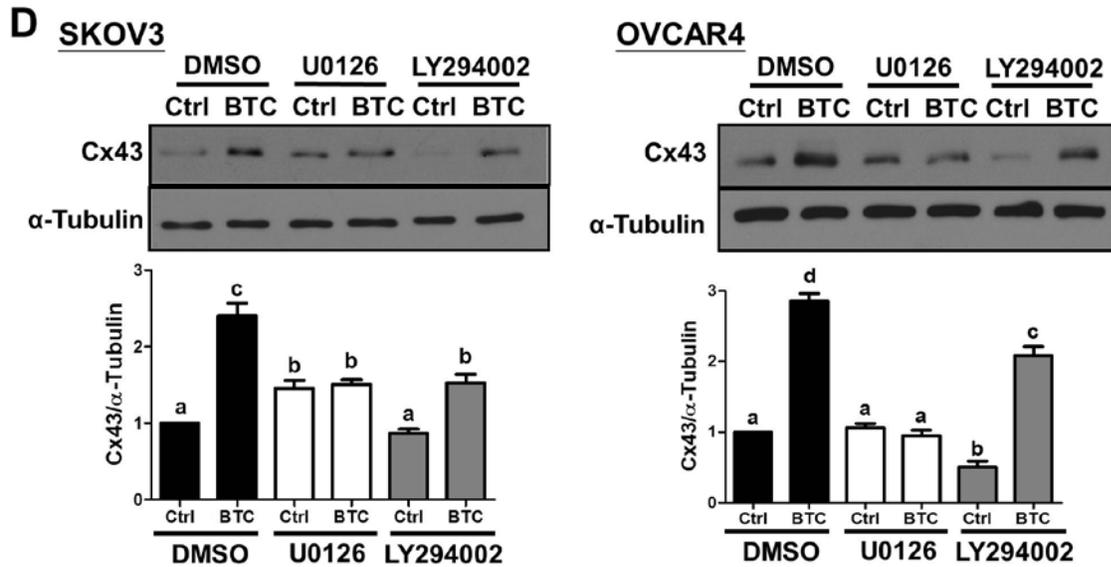
**A**, OVCAR4 and SKOV3 cells were treated without (Ctrl) or with 100ng/ml BTC for 1, 3, 6, 12 and 24 hours, and Cx43 mRNA levels were examined by RT-qPCR. **B**, Cells were treated without (Ctrl) or with 100ng/ml BTC for 3, 6, 12 or 24 hours, and Cx43 protein levels were examined by Western blot. **C-D**, Cells were treated for 24 hours without (Ctrl) or with increasing concentrations of BTC (1, 10, 20 or 50 ng/ml), and Cx43 mRNA (**C**) and protein (**D**) levels were examined 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 4.2. BTC up-regulates Cx43 partially via EGFR**

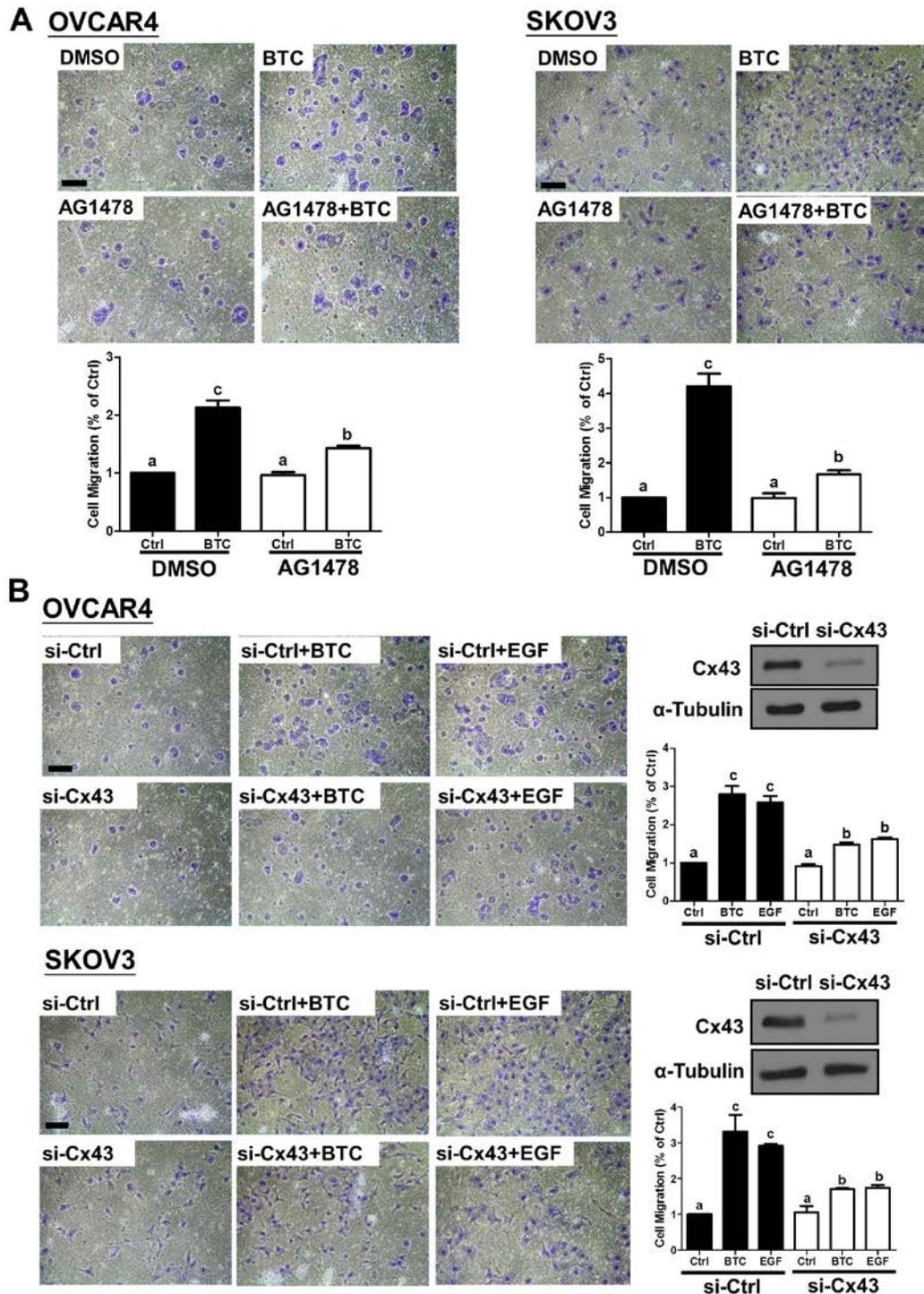
OVCAR4 and SKOV3 cells were pre-treated for 1 hour with vehicle control (DMSO) or 10  $\mu$ M AG1478 prior to treatment with or without 50 ng/ml BTC for 24 hours. Cx43 mRNA (A) and protein (B) levels were examined by RT-qPCR and Western blot, respectively. Results are expressed as the mean  $\pm$  SEM of at least three independent experiments and values without common letters are significantly different ( $P < 0.05$ ).





**Figure 4.3. MEK-ERK signaling mediates BTC-induced Cx43 expression**

**A**, SKOV3 cells were treated without (Ctrl) or with 50 ng/ml BTC for different time durations (10, 30 or 60 minutes), OVCAR4 cells were treated without (Ctrl) or with 50 ng/ml BTC or 50 ng/ml EGF for 30 minutes, and Western blots were used to measure the levels of phosphorylated Akt (p-Akt) and ERK1/2 (p-ERK1/2) in relation to their total levels (Akt and ERK1/2, respectively). **B**, OVCAR4 and SKOV3 cells were pre-treated for 1 hour with vehicle control (DMSO) or 10  $\mu$ M AG1478 prior to treatment with or without 50 ng/ml BTC for 30 minutes. Western blot was used to measure the Akt and ERK1/2 phosphorylation/activation. **C-D**, OVCAR4 and SKOV3 cells were pre-treated for 1 hour with vehicle control (DMSO), 5  $\mu$ M U0126 (MEK inhibitor) or 5  $\mu$ M LY294002 (PI3K inhibitor) prior to treatment with or without 50 ng/ml BTC for 24 hours. Cx43 mRNA (**C**) and protein (**D**) levels were examined 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 ( $P < 0.05$ ).

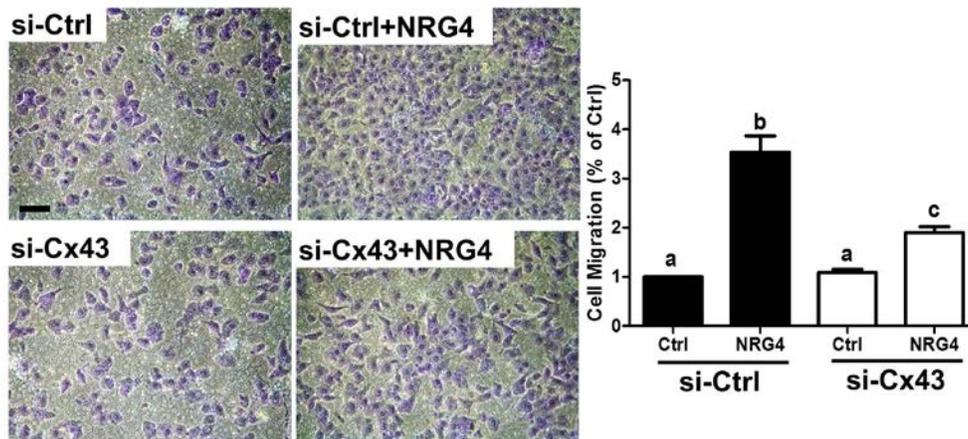


**Figure 4.4. Cx43 positively regulates BTC-induced ovarian cancer cell migration**

A, OVCAR4 and SKOV3 cells were pre-treated for 1 hour with vehicle control (DMSO) or 10  $\mu$ M AG1478 prior to treatment without (Ctrl) or with 50 ng/ml BTC, and cell migration was

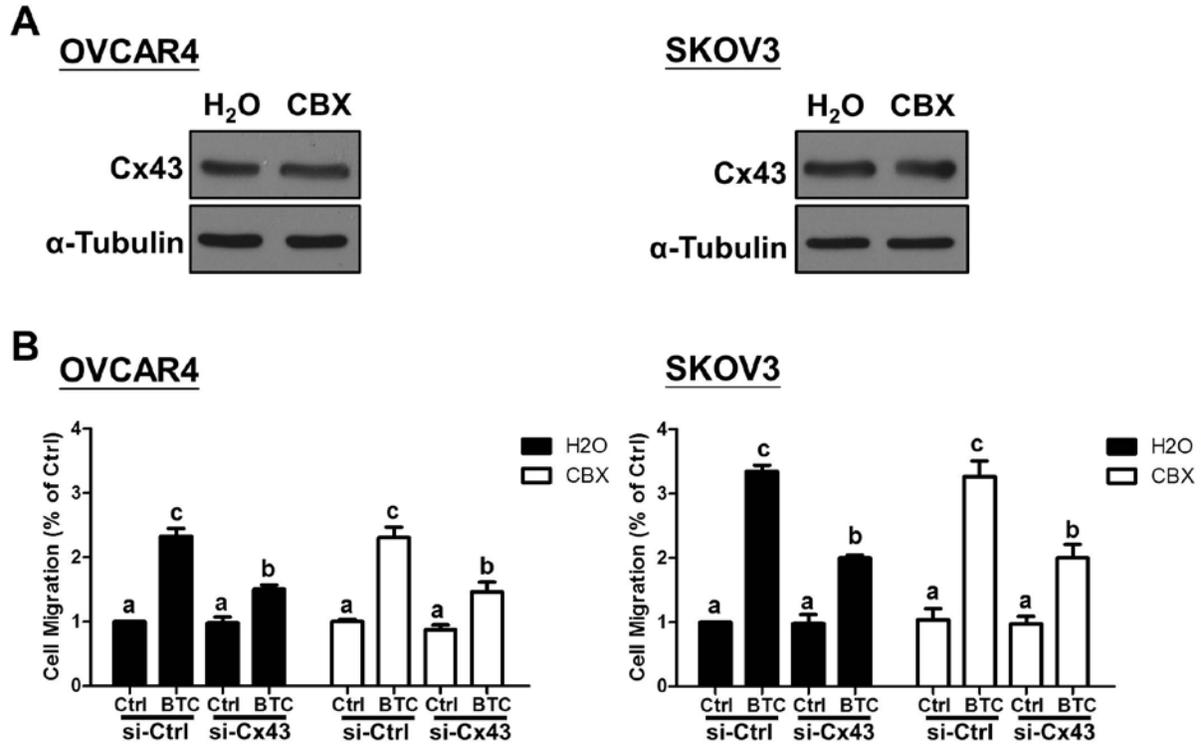
examined by transwell assay (24 hours). **B**, OVCAR4 and SKOV3 cells were transfected for 48 hours with 50 nM non-targeting control siRNA (si-Ctrl) or 50 nM siRNA targeting Cx43 (si-Cx43) prior to treatment without (Ctrl) or with 50 ng/ml BTC or 50 ng/ml EGF, and cell migration was examined by transwell assay (24 hours). Cells were stained with Crystal Violet; and pictures were taken under light microscope (10x objective) equipped with a digital camera. Results are expressed as the mean  $\pm$  SEM of at least three independent experiments and values without common letters are significantly different ( $P < 0.05$ ).

### SKOV3



**Figure 4.5. Neuregulin-4 induces SKOV3 cell migration via Cx43.**

SKOV3 cells were transfected for 48 hours with 50 nM non-targeting control siRNA (si-Ctrl) or 50 nM siRNA targeting Cx43 (si-Cx43) prior to treatment without (Ctrl) or with 10 ng/ml neuregulin-4 (NRG4). Cell migration was examined by transwell assay (12 hours). 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. BTC induces ovarian cancer cell migration in a gap junction-independent manner.**

A, OVCAR4 and SKOV3 cells were treated with vehicle control (H<sub>2</sub>O) or carbenoxolone (CBX; 25μM) for 48 hours and Cx43 protein levels were measured by Western blot. B, Cells were transfected for 24 hours with 50 nM non-targeting control siRNA (si-Ctrl) or 50 nM siRNA targeting Cx43 (si-Cx43), then pre-treated with CBX (25μM) for 1 hour prior to treatment without (Ctrl) or with 50 ng/ml BTC, and cell migration was examined by transwell assay (24 hours). Results are expressed as the mean ± SEM of at least three independent experiments and values without common letters are significantly different ( $P < 0.05$ ).

## **Chapter 5: Betacellulin promotes ovarian cancer cell proliferation via MEK-ERK, PI3K-Akt and CCN1 signaling**

### **5.1 Introduction**

Ovarian cancer is a life-threatening disease which takes away thousands of women's lives every year. Although the 5-year survival rate of ovarian cancer at all stages has increased from 36% to 46% due to incremental advances in existing therapeutic strategies, most ovarian cancer patients are diagnosed at late stages with distant metastasis and the 5-year survival remains lower than 30% [1]. Among all the factors that affect ovarian cancer oncogenesis and prognosis, the epidermal growth factor (EGF) family of ligands, together with their receptors and signaling pathways, have been shown to play important roles [50, 273, 274]. Betacellulin (BTC) is one of only three members of the EGF family capable of binding directly with EGFR and ERBB4, thereby enabling activation of all the possible combinations of ERBB dimers [62]. BTC was first identified in pancreatic  $\beta$ -cell tumors as a mitogen [67], and subsequent studies have investigated its involvement in several types of human cancer, including breast, endometrial and head and neck [76, 79, 80]. BTC mRNA has been detected in normal ovary and ovarian cancer, and our previous studies have shown that BTC induces ovarian cancer cell migration likely by down-regulating E-cadherin [275]. However, the roles of BTC in ovarian cancer cell growth and its potential mechanisms are completely unknown.

Cysteine-rich protein 61 (CYR61/CCN1) was the first member identified in the CCN family of matricellular proteins [200]. This family exerts a wide array of physiological functions by binding to various receptors, including integrins and low density lipoprotein receptor-related

proteins (LRPs) [182]. CCN1 has been implicated in the development and/or progression of multiple types of human cancer [181]. In particular, CCN1 mRNA and protein expression levels were significantly higher in epithelial ovarian tumors than benign ovarian tumors [209, 212, 213]. Immunohistochemical studies also showed that CCN1 overexpression was associated with advanced FIGO stage, poor differentiation and poor survival, making it an independent prognostic factor in these patients [212, 213]. Moreover, CCN1 has been shown to promote ovarian cancer cell proliferation and inhibit chemotherapy-induced apoptosis by modulating p53 and NF- $\kappa$ B expression [214-217]. To date, several factors have been shown to modulate CCN1 expression in ovarian cancer, including transcriptional co-activator TAZ and YAP [276, 277]. Our recent study also showed that S1P induced CCN1 expression by reducing YAP phosphorylation [218]. Knockdown of the E-cadherin transcriptional repressor Slug has been shown to reduce CCN1 expression [278]. However, whether BTC modulates CCN1 expression in ovarian cancer and the underlying mechanisms are completely unknown.

Based on these findings, we hypothesized that BTC can enhance ovarian cancer growth by up-regulating CCN1 expression. Our results show that BTC induces ovarian cancer cell proliferation by up-regulating CCN1 via EGFR. Moreover, BTC-induced cell proliferation is mediated by both MEK-ERK and PI3K-Akt signaling pathways. However, the signaling involved in BTC-increased CCN1 expression is still unclear and requires further investigation.

## **5.2 Materials and methods**

### **Cell culture**

SKOV3 and OVCAR3 human epithelial ovarian cancer cell lines were obtained from American Type Culture Collection. Cells were incubated in a 1:1 (vol/vol) mixture of

M199/MCDB105 medium (Sigma-Aldrich) supplemented with 10% (vol/vol) fetal bovine serum (FBS; Hyclone Laboratories) and 1% (vol/vol) penicillin/streptomycin (Gibco). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> and 95% air, and were serum starved for 24 hours prior to treatment.

### **Antibodies and reagents**

Monoclonal anti- $\alpha$ -tubulin (B-5-1-2) and polyclonal anti-Cyr61 (H-78) antibodies were from Santa Cruz Biotechnology. The horseradish peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were obtained from Bio-Rad Laboratories. *E. coli*-derived recombinant human betacellulin (Asp32-Tyr111) was obtained from R&D Systems and diluted in PBS. AG1478 and LY294002 were obtained from Sigma-Aldrich and diluted in DMSO. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma-Aldrich. U0126 was obtained from Calbiochem and diluted in DMSO.

### **Small interfering RNA (siRNA) transfection**

To knock down endogenous CCN1, cells were plated at low density, allowed to recover for 24 hours, and then transfected with 50 nM ON-TARGET*plus*SMARTpool siCCN1 (Dharmacon) using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. ON-TARGET*plus* non-targeting control pool siRNA (50 nM; Dharmacon) was used as a transfection control in all experiments.

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

Total RNA was extracted using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription was performed with 3 µg of RNA, random primers, and Moloney murine leukemia virus reverse transcriptase (Promega). SYBR Green RT-qPCR was performed on Applied Biosystems 7300 Real-Time PCR System equipped with 96-well optical reaction plates. Each 20 µl RT-qPCR reaction contained 1×SYBR Green PCR Master Mix (Applied Biosystems), 20 ng cDNA and 150 nM of each specific primer. The primers used were: *CYR61*, 5'-AGC CTC GCA TCC TAT ACA-3' (sense) and 5'-TTC TTT CAC AAG GCG GCA-3' (antisense); and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), 5'-GAG TCA ACG GAT TTG GTC GT-3' (sense) and 5'-GAC AAG CTT CCC GTT CTC AG-3' (antisense). The amplification parameters were 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. At least three separate experiments were performed on different cultures and each sample was assayed in triplicate. A mean value was used for the determination of mRNA levels by the comparative Cq method ( $2^{-\Delta\Delta Cq}$ ) with GAPDH as the reference gene.

### **Western blots**

Cells were lysed in lysis buffer (Cell Signaling Technology) containing protease inhibitor cocktail (Sigma-Aldrich). Lysates were centrifuged at 20,000 ×g for 10 minutes at 4°C and supernatant protein concentrations were quantified using the DC Protein Assay (Bio-Rad Laboratories) with BSA (A4503, Sigma-Aldrich) as the standard. 40 µg of protein was loaded in each well. Proteins were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes. After blocking with Tris-buffered saline containing 5% non-fat dry milk for 1 hour,

membranes were incubated overnight at 4°C with primary antibodies Cyr61 (1:2000), followed by incubation with the peroxidase-conjugated secondary antibody (1:5000) for 1 hour at room temperature. Immunoreactive bands were detected with enhanced chemiluminescent or SuperSignal West Femto substrate (Pierce). Membranes were stripped with stripping buffer (50 mM Tris-HCl pH 7.6, 10 mM  $\beta$ -mercaptoethanol and 1% SDS) at 50°C for 30 minutes and reprobed with anti- $\alpha$ -Tubulin as a loading control. Immunoreactive band intensities were quantified by densitometry, normalized to those of the relevant loading control, and the results are expressed as fold change relative to the respective control.

### **MTT assay**

Cell viability was examined using the MTT assay. SKOV3 and OVCAR3 cells were seeded in 96-well plates ( $1 \times 10^4$  cells per well; 200  $\mu$ l) and the next day treated with serum-free medium containing different concentrations of BTC for 24, 48, 72 or 96 hours. MTT was added at different time points to a final concentration of 0.5 mg/ml and then incubated for 4 h at 37 °C. Medium was removed, 100  $\mu$ l of DMSO was added to each well to dissolve the formazan crystal, plates were shaken gently for 10 min in the dark, and absorbances were read at 490 nm using a microplate reader (DYNEX technologies).

### **Statistical analysis**

Results are presented as the mean  $\pm$  SEM of at least three independent experiments. PRISM software (GraphPad Software Inc.) was used to perform one-way ANOVA followed by Tukey's multiple comparison test. Means were considered significantly different if  $P < 0.05$  and are indicated by different letters.

### **5.3 Results**

#### **BTC increases ovarian cancer cell viability**

To investigate the role of BTC in ovarian cancer cell growth and proliferation, we first treated two human ovarian cancer cell lines (SKOV3 and OVCAR3) with 100 ng/ml BTC for different periods of time (24-96 hours) and measured cell viability using MTT assay. As shown in Figure 5.1A, BTC increased the viability of both cell lines and these effects were most pronounced at 72 hours and of greater magnitude in SKOV3 cells. Next, SKOV3 and OVCAR3 cells were treated with varying concentrations of BTC (10-100 ng/ml) for 72 hours, and the results showed that 10 ng/ml BTC was sufficient to induce significant increases in viability in both cell lines (Figure 5.1B).

#### **BTC-increases ovarian cancer cell viability via EGFR-mediated activation of MEK-ERK and PI3K-Akt signaling**

The EGFR-specific inhibitor AG1478 was used to examine the role of EGFR in BTC-induced ovarian cancer cell proliferation. As shown in Figure 5.2A, pre-treatment with AG1478 totally blocked BTC-induced increases in cell viability in both SKOV3 and OVCAR3 cells. Next, we used the MEK inhibitor U0126 and the PI3K inhibitor LY294002 to investigate the involvement of these two common EGF-related signaling pathways in BTC-induced cell proliferation. The results show that pre-treatment with U0126 almost completely blocked the effects of BTC on SKOV3 and OVCAR3 cell viability (Figure 5.2B). In contrast, pre-treatment with LY294002 partially reduced the effects of BTC in OVCAR3 cells but did not seem to alter BTC effects in SKOV3 cells (Figure 5.2B).

### **BTC up-regulates CCN1 expression in ovarian cancer cells**

To investigate the effects of BTC on CCN1 expression, we first measured CCN1 mRNA levels in SKOV3 and OVCAR3 cells following treatment with BTC (100 ng/ml) for varying times (1, 3, 6, 12 or 24 hours). RT-qPCR results showed that BTC rapidly up-regulated CCN1 mRNA levels, with the greatest effect occurring 1 hour after treatment (Figure 5.3A). Western blot was used to confirm the up-regulation of CCN1 production at the protein level (Figure 5.3B). Next, we examined the concentration-dependent (1-50 ng/ml) effects of BTC on CCN1 mRNA and protein levels. As shown in Figure 5.3C and 5.3D, treatment for 1 hour with varying concentrations of BTC induced concentration-dependent increases in CCN1 mRNA and protein levels in both SKOV3 and OVCAR3 cells. It is worth noting that both cell lines showed very high sensitivities to BTC with respect to induction of CCN1 expression.

### **EGFR, but not MEK-ERK or PI3K-Akt signaling, is required for BTC-induced CCN1 expression**

Specific inhibitors were used to explore the involvement of EGFR, MEK-ERK and PI3K-Akt signaling in the up-regulation of CCN1 production by BTC. As shown in Figure 5.4A and 5.4B, pre-treatment with the EGFR inhibitor AG1478 abolished the up-regulation of CCN1 mRNA and protein levels by BTC in both SKOV3 and OVCAR3 cells. However, neither the MEK inhibitor U0126 nor the PI3K inhibitor LY294002 markedly affected BTC-induced increases in CCN1 mRNA and protein levels (Figure 5.4C and 5.4D).

## **CCN1 mediates the effects of BTC on ovarian cancer cell viability**

To determine whether or not CCN1 is involved in BTC-induced ovarian cancer cell proliferation, we used CCN1 specific siRNA to knockdown endogenous CCN1 in SKOV3 cells. MTT assay results showed that pre-treatment with si-CCN1 partially reversed the effect of BTC on SKOV3 cell viability (Figure 5.5).

## **5.4 Discussion**

BTC was initially identified as a mitogen in mouse pancreatic  $\beta$ -cell tumor cells [62]. Although the specific role of BTC in tumor growth and development remains poorly defined, several studies have confirmed the growth-stimulatory effects of BTC in normal tissues. For example, BTC has been shown to promote  $\beta$ -cell proliferation and differentiation, leading to improved glucose tolerance [279-281]. BTC has also been suggested to induce urothelial [282], osteoblastic [258], neural stem cell [283] and intestinal epithelium proliferation [284], with the last study demonstrating that BTC overproduction increased the number of polyps rising in *Apc<sup>+Min</sup>* mice [284]. Our results demonstrate for the first time a role for BTC in the promotion of ovarian cancer cell viability, with CCN1 as a target gene for these growth-promoting effects. Further study is needed to fully characterize this function.

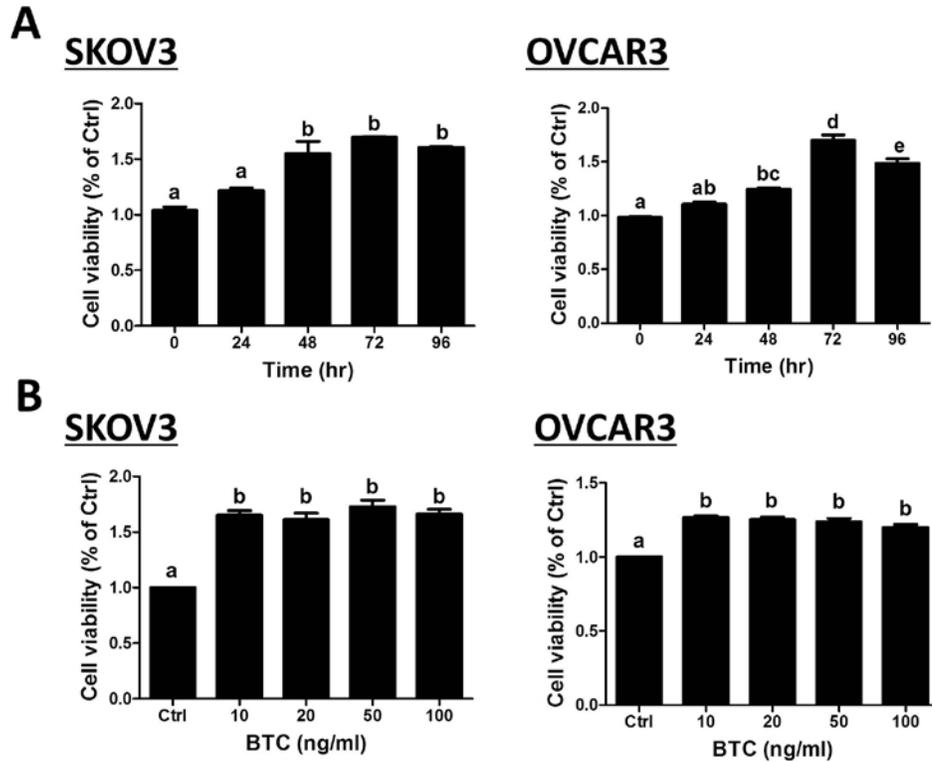
BTC is distinct from EGF in many aspects because it can bind with both EGFR and ERBB4 [62]. We have already shown that BTC-induced ovarian cancer migration is only partially mediated by EGFR [262], whereas the present results suggest that BTC-induced ovarian cancer cell proliferation is exclusively mediated by EGFR. Future studies are required to confirm these findings; however they suggest function-specific involvement of EGFR vs. ERBB4 in BTC signaling. Several studies are in agreement with the notion that BTC-induced cell proliferation is

mainly mediated by EGFR, possibly in combination with ERBB2 [72, 246]. However, other studies suggest that ERBB4 can mediate BTC-induced cell proliferation. For example, BTC has been shown to maintain extravillous trophoblast growth via ERBB4 in term placentas [285], and to induce expansion of neuroblasts via ERBB4 [283]. In addition, studies in transgenic mice suggest that BTC-induced urothelial hyperplasia is not exclusively dependent on EGFR [282]. Taken together, activation of different ERBB receptors by BTC may be differentially coupled to specific cellular functions in ovarian cancer. Thus, whether or not ERBB4 is involved in BTC-induced ovarian cancer cell proliferation warrants further investigation.

PI3K-Akt and MEK-ERK are two of the most common ERBB downstream signaling pathways [54]. Our previous studies have shown that BTC can modulate E-cadherin and Cx43 expression via these two signaling pathways [275]. However in the present study, inhibition of MEK or PI3K had no effect on the up-regulation of CCN1 by BTC. These results suggest that although MEK-ERK and PI3K-Akt signaling have been shown to modulate CCN1 production [286, 287], they are not involved in BTC-induced CCN1 expression in ovarian cancer. Several other pathways have been shown to modulate CCN1 expression and we may find some potential connection with BTC signaling [201]. For instance, RhoA GTPases and p38 MAPK pathways have been implicated in the induction of CCN1 in smooth muscle cells and endothelial cells [203]. BTC has been shown to regulate the activity of another member of the Rho GTPase family, Rac1, and control actin remodeling and islet migration [245]. Unfortunately, although p38 MAPK can be phosphorylated by BTC in smooth muscle cells [72], it was not activated in our cell line models (data not shown). In prostate cancer cells, CCN1 is positively regulated by cAMP/PKA signaling [288]; while the anti-apoptotic effect of BTC in islets is partially mediated by cAMP response element binding protein [289]. In addition, Klein *et al.* demonstrated that

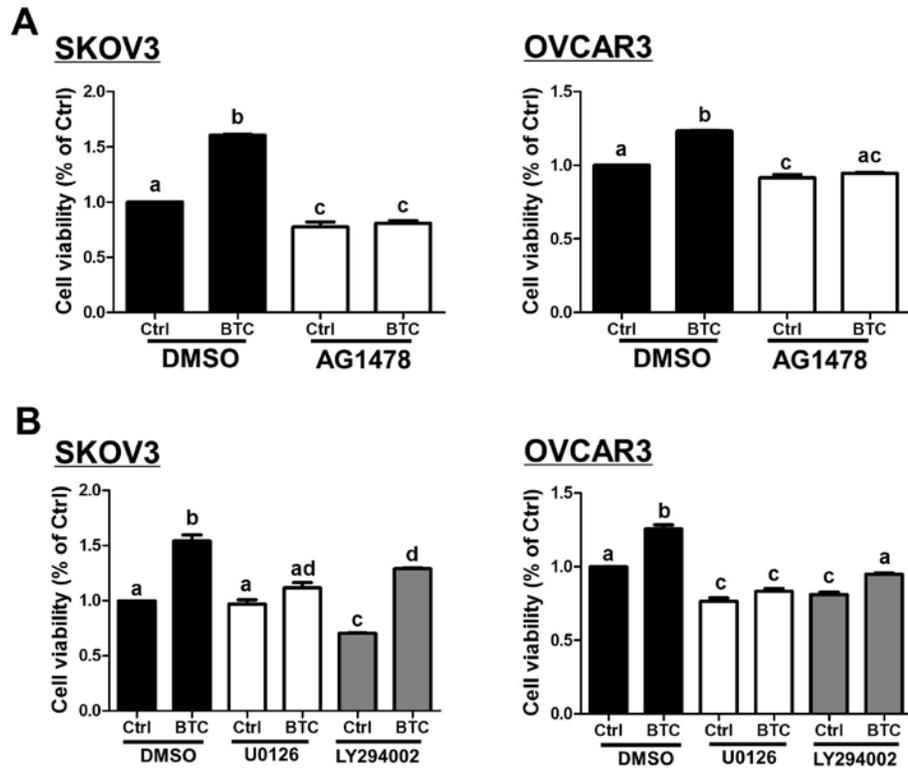
inhibition of MEK-ERK signaling could not block EGF-induced CCN1 up-regulation in endometrial epithelial cells [202]. Instead, they showed that EGF up-regulates CCN1 expression via JAK2/STAT3 signaling. Interestingly, BTC has been shown to activate STAT3 in HeLa cells [290]. In addition to the signaling pathways discussed above, early growth response gene product (Egr)-1, Wnt signaling and hypoxia inducible factor (HIF)-1 $\alpha$  have also been shown to modulate CCN1 production [201, 291, 292]. Future studies aimed at clarifying the mechanism by which BTC up-regulates CCN1 expression in ovarian cancer will be of interest.

In summary, our study demonstrates that BTC promotes ovarian cancer cell proliferation via EGFR. This process is mainly mediated by MEK-ERK and less mediated by PI3K-Akt signaling. In addition, CCN1 is up-regulated by BTC via EGFR and positively mediates BTC-induced cell proliferation, although the underlying signaling requires further investigation.



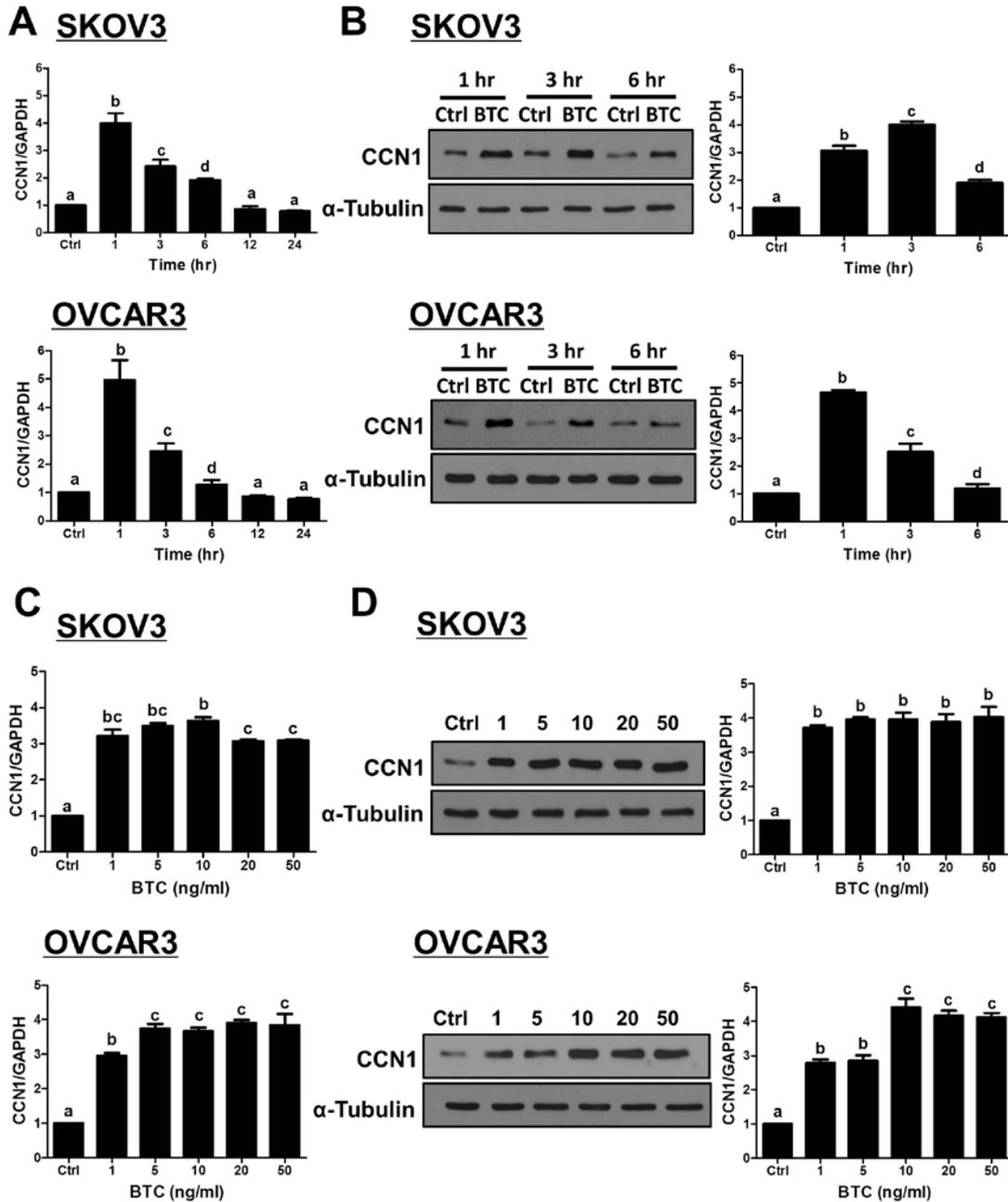
**Figure 5.1. BTC induces ovarian cancer cell proliferation.**

**A.** SKOV3 and OVCAR3 cells were treated without (Ctrl) or with betacellulin (BTC: 100 ng/ml) for 24, 48, 72 or 96 hours, and cell viabilities were examined by MTT assay. **B.** SKOV3 and OVCAR3 cells were treated for 72 hours without (Ctrl) or with increasing concentrations of betacellulin (BTC: 10, 20, 50 or 100 ng/ml), and cell viabilities were examined by MTT assay. 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 5.2. BTC-induced ovarian cancer cell proliferation involves EGFR-mediated activation of MEK-ERK and PI3K-Akt signaling.**

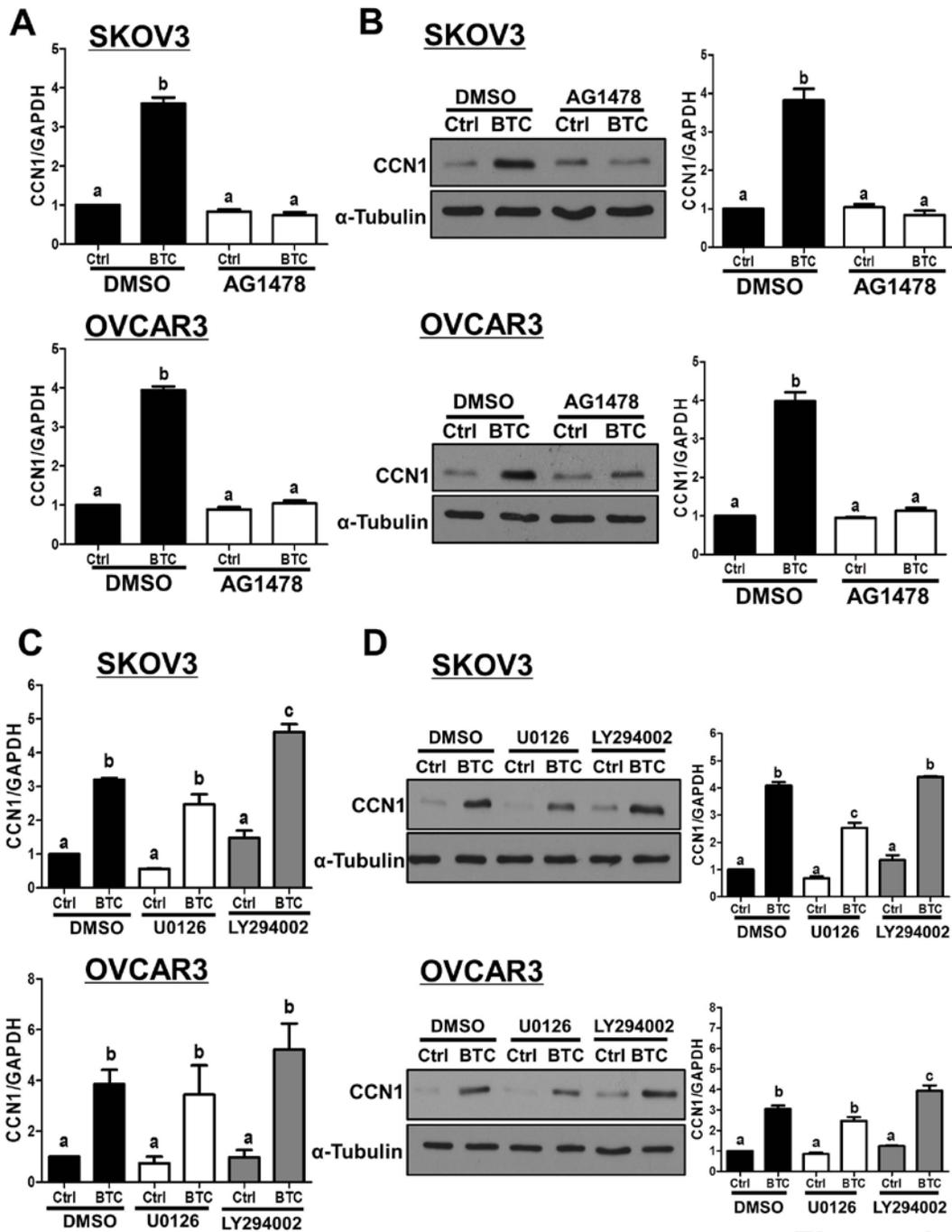
**A.** SKOV3 and OVCAR3 cells were pre-treated for 1 hour with vehicle control (DMSO) or 5  $\mu$ M AG1478 prior to treatment with or without 10 ng/ml BTC for 72 hours. Cell viabilities were examined by MTT assay, respectively. **B.** SKOV3 and OVCAR3 were pre-treated for 1 hour with vehicle control (DMSO), 5  $\mu$ M U0126 (MEK inhibitor) or 5  $\mu$ M LY294002 (PI3K inhibitor) prior to treatment with or without 10 ng/ml BTC, and cell viabilities were examined by MTT assay. 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 5.3. BTC up-regulates CCN1 expression in ovarian cancer cells.**

**A-B.** SKOV3 and OVCAR3 cells were treated without (Ctrl) or with 100 ng/ml BTC for 1, 3, 6, 12 or 24 hours. A, CCN1 mRNA levels were examined by RT-qPCR. B, CCN1 protein levels were examined by Western blot. **C-D.** SKOV3 and OVCAR3 cells were treated for 1 hour

without (Ctrl) or with increasing concentrations of betacellulin (BTC: 1, 5, 10, 20 or 50 ng/ml), and CCN1 mRNA (**C**) and protein (**D**) levels were examined 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 ( $P<0.05$ ).

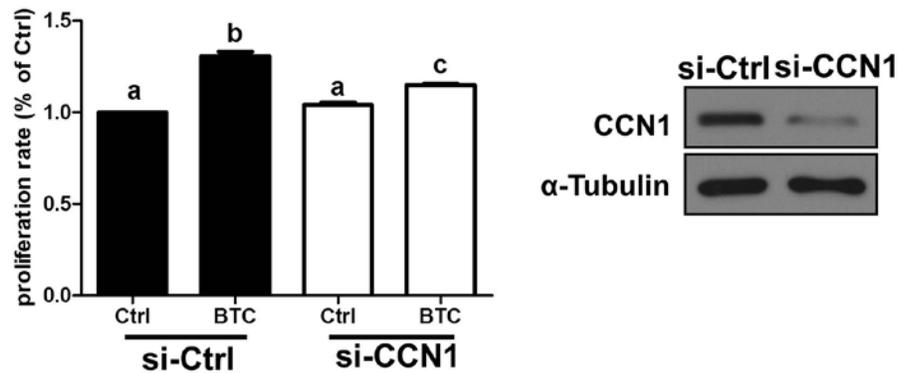


**Figure 5.4. EGFR, but not MEK-ERK or PI3K-Akt pathway, is required for BTC-induced CCN1 expression.**

**A-B.** SKOV3 and OVCAR3 cells were pre-treated for 1 hour with vehicle control (DMSO) or 5  $\mu$ M AG1478 prior to treatment with or without 10 ng/ml BTC for 1 hours. CCN1 mRNA (A) and

protein (**B**) levels were examined by RT-qPCR and Western blot, respectively. **C-D**. SKOV3 and OVCAR3 were pre-treated for 1 hour with vehicle control (DMSO), 5  $\mu$ M U0126 (MEK inhibitor) or 5  $\mu$ M LY294002 (PI3K inhibitor) prior to treatment with or without 10 ng/ml BTC, and CCN1 mRNA (**C**) and protein (**D**) levels were examined 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 ( $P<0.05$ ).

## SKOV3



**Figure 5.5. CCN1 mediates BTC-induced ovarian cancer cell proliferation.**

SKOV3 cells were transfected for 48 hours with 50 nM non-targeting control siRNA (si-Ctrl) or 50 nM siRNA targeting CCN1 (si-CCN1) prior to treatment for 72 hours without (Ctrl) or with 10 ng/ml betacellulin (BTC). CCN1 knockdown efficiency were examined by Western blot and cell proliferation was measured by MTT assay. Results are expressed as the mean  $\pm$  SEM of at least three independent experiments and values without common letters are significantly different ( $P < 0.05$ ).

## Chapter 6: Conclusion

### 6.1 Summary

Ovarian cancer is the most lethal gynecological malignancy in developed countries [1]. Although extensive studies have been done to develop early detection methods and new therapeutic strategies, more than 60% of ovarian cancer patients are still diagnosed with distant metastasis and their 5-year survival rate remains lower than 30% [1]. The roles of EGF family ligands and receptors in cancer progression have been studied in many types of human tumors including ovarian cancer. However, much of this research has been focused on prototypical members (e.g. EGF) and much less is known about the roles of BTC in ovarian cancer development, growth and metastasis. In the current thesis, we investigate the biological functions of BTC in ovarian cancer proliferation and migration; and our results could inform new therapeutic strategies targeting BTC ligand together with its signaling for ovarian cancer treatment.

In chapter 3, the effects of BTC on ovarian cancer cell migration and EMT, combined with underlying mechanism were studied. BTC induced SKOV3 and OVCAR5 cell migration significantly and down-regulated E-cadherin; MEK-ERK and PI3K-Akt signaling pathways mediated these effects. In addition, EGFR specific inhibitor AG1478 totally blocked BTC-induced signaling activation and E-cadherin down-regulation, and partially reversed BTC-induced cell migration, while pan-ERBB inhibitor AST1306 totally inhibited BTC-induced cell migration. Importantly, although BTC treatment increased both Snail and Slug expression, only Slug was involved in E-cadherin transcriptional repression by BTC. This study demonstrated for

the first time that BTC may promote EMT-like changes and enhances ovarian cancer cell migration.

In chapter 4, we investigated if a crucial gap-junction component, Cx43, is involved in BTC-induced ovarian cancer cell migration. Our results showed that BTC up-regulated Cx43 expression mainly via MEK-ERK signaling and partially via EGFR. In addition, NRG4, which only bind to ERBB4, induced SKOV3 cell migration. More importantly, knockdown of endogenous Cx43 using specific siRNA partially attenuated BTC and NRG4-induced SKOV3 and OVCAR4 cell migration. This study demonstrated a positive effect of Cx43 on ovarian cancer cell migration. Taken together with previous findings, Cx43 may exert dual roles in ovarian cancer development and progression. In addition, our results suggest the involvement of ERBB4 in BTC-induced ovarian cancer migration but further confirmation is required.

In chapter 5, we tested the role of BTC in ovarian cancer cell viability and confirmed proliferative effects of BTC in SKOV3 and OVCAR3 cells. This effect was fully mediated by EGFR through MEK-ERK and PI3K-Akt signaling pathways. We also demonstrated that BTC increased production of the matricellular protein CCN1; and knockdown of CCN1 partially reversed BTC-enhanced cell proliferation. This study examined another role of BTC in ovarian cancer cell activities and further suggests the importance of BTC signaling in ovarian cancer oncogenesis and development. A schematic diagram of the results of the present studies are presented in Figure 6.1.

## **6.2 Discussion**

### **6.2.1 The importance of ERBB4 in ovarian cancer progression and its potential involvement in BTC signaling**

Like the prototype EGFR, ERBB4 has both ligand-binding ability and tyrosine-kinase activity [250]. However, unlike other members of the ERBB family, the function of ERBB4 is largely determined by alternative splicing [293]. In spite of its unique properties, not very much is known about ERBB4 signaling in cancer biology, especially with regards to ovarian cancer. Several groups have observed elevated expression of ERBB4 in ovarian cancer, with expression levels in malignant tumors being significantly higher than in benign tumors or normal ovary [251, 254, 255]. A recent study has indicated that high-level ERBB4 expression is correlated with platinum resistance and reduced overall survival in ovarian cancer [294]. In addition, a specific isoform of ERBB4 (CYT-1) increases cell growth and is an independent prognostic factor in serous ovarian cancer [256]. These findings suggest the clinical importance of ERBB4 in ovarian cancer.

In Chapter 3, we showed that EGFR inhibitor AG1478 only partially attenuated BTC-induced cell migration in SKOV3 and OVCAR5, while the pan-ERBB inhibitor AST1306 fully blocked BTC-induced cell migration (Figure 3.8). Since ERBB2 does not bind any known ligand, this result suggests the involvement of ERBB4 in BTC-induced ovarian cancer cell migration. In Chapter 4, we treated cells with neuregulin-4 to further investigate the role of ERBB4 in ovarian cancer cell migration. As shown in Figure 4.5, neuregulin-4 significantly increased SKOV3 cell migration and knockdown of endogenous Cx43 partially attenuated this effect. These results implicate ERBB4 signaling in ovarian cancer cell migration and suggest the involvement of

Cx43, which could explain why pre-treatment with the EGFR inhibitor AG1478 only partially reduced BTC-induced Cx43 expression (Figure 4.2).

Taken together, clarifying the biological effects of ERBB4 and its role in BTC signaling could help to uncover novel molecular pathways in ovarian cancer metastasis and chemotherapy resistance, which may further contribute to targeting treatment.

### **6.2.2 What are the genomic profiles of my cell models and how do they relate to the differential response of different cell lines to BTC treatment?**

Established cancer cell lines are frequently used as *in vitro* tumor models to study cancer biology. However, cancer cell lines may not fully represent the nature of primary tumors *in vivo*. In order to minimize the gap between cell lines and real tumors, suitable cell lines should be chosen based on their genetic profiles similarity to specific tumor subtypes. In our studies, we used four epithelial ovarian cancer cell lines, SKOV3, OVCAR5, OVCAR4 and OVCAR3 to conduct the experiments. Recent studies have suggested that OVCAR3 and OVCAR4 are suitable cell models for HGSC, whereas OVCAR5 may not be a HGSC cell line and SKOV3 is considered to be of clear cell- or endometrioid-like origin [223, 224]. Thus, our studies were not restricted to a single EOC subtype. We used two of these four EOC cell lines in each research section and our results showed that each cell line responded differentially upon BTC treatment. For instance, SKOV3 cells displayed greater responses to BTC with respect to cell migration, proliferation and target gene expression than the other three cell lines; it also had differences in the importance of PI3K signaling for Cx43 and proliferation. These differences could reflect histotype-specific roles of BTC on EOC cell activities or they may be unique to a given cell line.

Future studies testing more EOC cell lines of differing histotype would help to resolve whether there are histotype-specific differences in the effects of BTC on EOC cells.

Domcke *et al.* have shown that SKOV3 has amplification of ERBB2, which is in agreement with our Western blot results of ERBB receptors expression levels in each cell line (Figure 3.2). ERBB2 is the favorite partner for other ERBB receptors and amplifies responses by activating numerous signaling cascades [295]. Overexpression of ERBB2 has been found in many types of human cancers, and has been shown to associate with advanced malignancy and poor prognosis in EOC [296, 297]. Differences in the response of SKOV3 cells to BTC might therefore result from the amplification of ERBB2, and further suggests that responses to BTC could be influenced by the relative expression of ERBB receptors (EGFR, ERBB2 and ERBB4). Interestingly, ERBB2 amplification/overexpression is observed in ~19% of mucinous ovarian cancers but the role of BTC is completely unknown in this subtype [298].

Taken together, clarifying the molecular basis of differential responses of cell lines to BTC treatment and pharmacological inhibitors as well as choosing suitable cell models for different subtypes will be of great importance for future studies of BTC function in EOC.

### **6.2.3 Is there any cross-talk between BTC-regulated E-cadherin, Cx43 and CCN1?**

In the current study, we confirmed separately that BTC induces ovarian cancer migration by down-regulating E-cadherin and up-regulating Cx43, and promotes cell proliferation by increasing CCN1 expression. However, *in vivo* BTC will affect tumor cell activities by modulating multiple signaling pathways and target genes synchronously. In that case, what is the cross-talk among BTC-regulated E-cadherin, Cx43 and CCN1 in ovarian cancer migration and proliferation?

CCN1 has been shown to induce EMT and cell metastasis by suppressing E-cadherin expression in oncogenesis. In laryngeal tumors, E-cadherin expression was significantly higher in CCN1 negative tumors than in CCN1 positive tumors [299]. Overexpression of CCN1 also induced cell proliferation and invasion by down-regulating E-cadherin in breast cancer [300] and prostate cancer [301]. In addition, CCN1 induced esophageal epithelium to progress towards esophageal adenocarcinoma by reducing E-cadherin expression and disrupting cell-cell junctions [302]. Cross-talk between CCN1 and E-cadherin is likely to involve  $\beta$ -catenin, which is crucial for E-cadherin-maintained cell adhesion.  $\beta$ -catenin links the cadherin complex with the actin cytoskeleton and helps to establish the polarity and epithelial phenotype of epithelial cells [303]. On the other hand, canonical Wnt/ $\beta$ -catenin pathway is also essential for CCN1 induction and signal transduction [201]. Overexpression of CCN1 results in the accumulation and translocation of  $\beta$ -catenin into the nucleus [304, 305], where  $\beta$ -catenin binds with T-cell factor/lymphocyte-enhancing factor and works as a transcriptional regulator [306]. This sequestration of  $\beta$ -catenin in nucleus directly promotes EMT [307], and is also associated with loss of E-cadherin expression, which further attenuates cell adhesion and increase the susceptibility of epithelial tumor cell to enter EMT [308, 309]. In our study, BTC oppositely regulates E-cadherin and CCN1 expression. Although we do not test the direct correlation between up-regulation of CCN1 and down-regulation of E-cadherin, our result is in agreement with the evidence discussed above, and suggests synergetic roles of E-cadherin and CCN1 in BTC-induced ovarian cancer cell migration. Future studies investigating the involvement of CCN1 in BTC-induced ovarian cancer cell migration and if CCN1 can inhibit E-cadherin expression and induce EMT will be of great interest.

Adherens junctions and gap junctions are two major types of cell junctions. Contact of neighbor cells is the prerequisite of channel communication; and they also have collaborative effect in anchoring cytoskeleton and preserving epithelial cell polarity and phenotype. As well-known members of these two cell junctions, E-cadherin and Cx43 share many characteristics on molecular level: they both can directly or indirectly bind with microtubules and actin and regulate their stability; they can recruit  $\beta$ -catenin and zonula occludens-1 to the plasma membrane and prevent their transcriptional activities; and they can be modulated by tyrosine phosphorylation [128]. In oncogenesis, reduced expression and positive correlation between E-cadherin and Cx43 has been observed in laryngeal carcinomas [310], endometrioid endometrial adenocarcinoma [311] and non-small cell lung cancer [312]. In addition, studies have shown that E-cadherin and Cx43 can modulate each other's expression. For example, overexpression of Cx43 significantly induced E-cadherin expression in lung cancer [313, 314] and glioma stem cells [315]; whereas E-cadherin expression facilitated the assembly of Cx43 in liver epithelial cells [316] and human squamous carcinoma [317]. In our study, E-cadherin and Cx43 are oppositely involved in BTC-induced ovarian cancer cell migration, which leads us to speculate their connection from a different aspect. There is one study demonstrating that high Cx43 expression is directly related with high invasiveness and EMT-related phenotype in prostate cancer cells. They show that overexpression of Snail induces Cx43 expression, and the feedback between Snail and Cx43 determines the invasive potential of cancer cells [318]. It is tempting to speculate that although Snail is not involved in BTC-reduced E-cadherin in ovarian cancer cells, it may participate in BTC-induced Cx43 expression; and these molecules may work together to promote EMT and cell migration. Future studies clarifying whether Snail or Slug can modulate

BTC-induced Cx43 expression and the role of Cx43 in E-cadherin expression and EMT in ovarian cancer cell will be of great interest.

Besides its role in modulating cell adhesion and motility, Cx43 is also known for regulating cell growth and proliferation under multiple conditions [319]. The growth inhibition effect of Cx43 is partially due to the permeability of gap junction, and can also be modulated in a GJIC-independent manner [319]. As mentioned above, Cx43 can sequester  $\beta$ -catenin and zonula occludens-1 to the plasma membrane, minimize their down-stream signaling and maximize growth inhibition. Cx43 is also positively related with CCN3, another CCN family member that is usually induced by growth inhibition and has anti-proliferative effects [320, 321]. The expression of Cx43 induces CCN3 expression and induces redistribution of CCN3 from the cytoplasm to the plasma membrane where it co-localizes with gap junctions [321]. In our study, BTC induces ovarian cancer cell proliferation by up-regulating CCN1, and our previous study just showed that EGF-induced Cx43 negatively regulates cell proliferation in a GJIC-independent way. Does Cx43 work similarly in BTC-induced cell proliferation? If so, is there any interaction between CCN1 and Cx43 in oppositely modulating BTC-induced cell proliferation?

Taken together, the correlation between E-cadherin, Cx43 and CCN1 in EMT, cell migration and proliferation is complex. Making clear the cross-talk among them and how they work coordinately to mediate BTC function will help us better understand the signaling of BTC and further clarify the roles of BTC in ovarian cancer cell activities.

#### **6.2.4 What is the potential role of BTC in ovarian cancer angiogenesis?**

Like other members of the EGF family, BTC has been shown to participate in various pathophysiological processes in addition to cell migration and proliferation [62]. Several studies have suggested a role for BTC in angiogenesis. BTC induced the growth and migration of different types of vascular smooth muscle cells through ERK1/2 and Akt signaling pathways; although the receptor(s) mediating these functions is not clear [72, 249]. In human head and neck squamous cell carcinomas, BTC modulated the expression of VEGF by activating EGFR and ERBB2, which could be one mechanism for BTC-induced angiogenesis [322]. In addition, overexpression of BTC in hepatocellular carcinoma cells combined with overexpression of EGFR in sinusoidal endothelial cells could also enhance angiogenesis in a paracrine manner [77].

On the other hand, the target genes we studied in the current thesis have also been shown to associate with angiogenesis in various conditions. CCN1 is a well-recognized angiogenic factor that enhances angiogenesis, vascular smooth muscle cell functions, and the production of angiogenic molecules in both normal vascular development and tumor biology [323]. Varying effects of Cx43 have also been described in angiogenesis. Reduced expression of Cx43 in vascular cells inhibits gap-junctional communication and leads to down-regulation of VEGF, reduced cell viability and impaired vascular remodeling [324-326]. Gap-junction independent functions of Cx43 in the promotion of angiogenesis have also been observed in mesenchymal stem cells [327]. However in human breast cancer and murine breast and skin tumors, Cx43 is suggested to suppress angiogenesis [328, 329]. Taken together, these findings combined with our results suggest a potential role of BTC in ovarian cancer angiogenesis and warrant future investigation.

### **6.2.5 What is the clinical application of BTC signaling in ovarian cancer treatment?**

Overexpression of ERBB family members have been widely documented in human cancers [330] and specific inhibitors and antibodies targeting ERBB members and signaling pathways have been extensively studied in the past decades [54]. These approaches have proved effective in several fatal cancers including lung cancer, breast cancer, gastric cancer and colorectal cancer [54]. However, some commonly used EGFR monoclonal antibodies and tyrosine-kinase inhibitors showed very limited clinical activities in ovarian cancer [331-334]. Perhaps targeting ERBB ligands (either with or without conventional receptor inhibition) could improve effectiveness in ovarian cancer treatment or be used for early detection.

HB-EGF is another EGFR/ERBB4 ligand in the EGF family and could be a good model for studying the clinical application of BTC in ovarian cancer [335, 336]. A specific HB-EGF inhibitor, cross-reacting material 197 (CRM197), was developed to block the binding of HB-EGF to its receptors. It was shown to attenuate multiple malignant phenotypes in ovarian cancer cells and to block all steps involved in ovarian cancer peritoneal dissemination [337]. In 2007, a clinical study of CRM197 in advanced ovarian cancer became the first approved ERBB-ligand-targeted therapy [338]. Besides CRM197, several anti-HB-EGF monoclonal antibodies such as Y-142 and KM3566 have also been shown to inhibit ovarian cancer progression and could be used to improve clinical outcome [339, 340]. Considering the numerous pathological functions of BTC and our demonstration of BTC effects on ovarian cancer cells, we have reasons to look forward to the future study of BTC in ovarian cancer oncogenesis and prognosis. Establishing a specific BTC antibody or inhibitor may provide new therapeutic strategies for ovarian cancer prevention and treatment.

### **6.3 Limitations of this study**

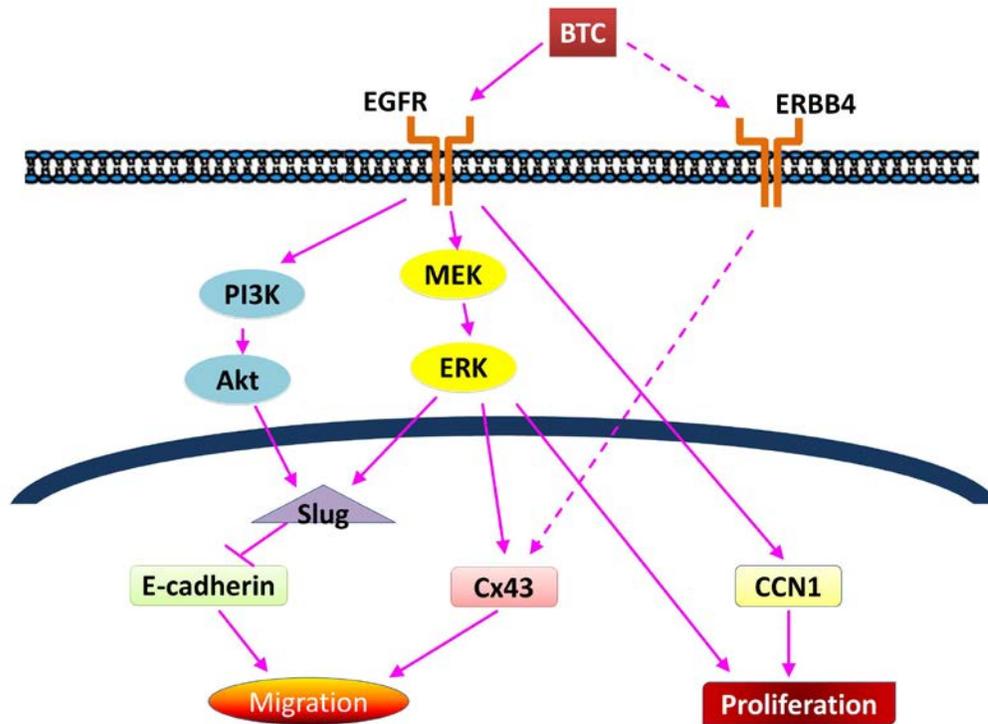
The limitation of current study is that we conducted all the experiments *in vitro*. Although the cell lines we chose, including SKOV3, OVCAR3, OVCAR4 and OVCAR5, have been widely used in ovarian cancer studies, they cannot fully represent the nature of the original tumor in the heterogeneous population of clinical patients. The growth and development of ovarian tumor *in vivo* can be affected by many other factors such as tumor microenvironment and signaling cross-talk. In that case, it will be of great value to repeat our study in feasible animal models, such as mouse xenograft model. If BTC can stimulate ovarian cancer growth or metastasis *in vivo*, then the value of targeting BTC signaling in treating ovarian cancer patients could be further verified.

### **6.4 Future directions**

- 1) Given the limitations of the current study, *in vivo* animal models should be used in the future to further confirm the role of BTC in ovarian cancer progression. To be specific, we can establish a stable overexpression of BTC in ovarian cancer cells, and inject the cells in BALB/c nude mice and monitor the growth and size of the xenograft tumor.
- 2) In the current study, we demonstrated that BTC induces ovarian cancer cell migration and proliferation mainly via EGFR. However, our results also suggest the potential involvement of ERBB4 in BTC-enhanced cell migration, which has not been well elucidated in any cancer yet. Thus, future studies clarifying ERBB4-mediated BTC signaling and downstream target genes in ovarian cancer cell activities will be of great value.
- 3) In the current study, we used several cell lines from different subtypes of epithelial ovarian cancer to conduct the experiments and we cannot rule out histotype specific effects of BTC.

Future studies with more cell lines could be used to further confirm the differential roles of BTC in different subtypes of epithelial ovarian cancer.

- 4) Besides cell migration and proliferation, alteration of E-cadherin, Cx43 and CCN1 have also been identified in other cancer cell activities such as invasion and angiogenesis. Therefore, the potential roles of BTC in ovarian cancer adhesion, invasion, angiogenesis and apoptosis will be worth studying in the future in order to better understand BTC features and provide additional information for clinical trials.



**Figure 6.1.** A schematic diagram of proposed mechanism of BTC modulating epithelial ovarian cancer migration and proliferation.

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