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Exploring the regulation and function of epithelial-mesenchymal plasticity in ovarian cancer spheroids

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Abstract

Epithelial-mesenchymal transition (EMT) serves as a key mechanism driving tumour cell migration, invasion, and metastasis in many carcinomas. Transforming growth factor-beta (TGFβ) signalling is implicated in several steps during cancer pathogenesis and acts as a classical inducer of EMT. Since epithelial ovarian cancer (EOC) cells have the potential to switch between epithelial and mesenchymal states during metastasis, we predicted that modulation of TGFβ signalling would significantly impact EMT and the malignant potential of EOC spheroid cells. Ovarian cancer patient ascites-derived cells naturally underwent an EMT response when aggregating into spheroids, and this was reversed upon spheroid re-attachment to a substratum. *CDH1*/E-cadherin expression was markedly reduced in spheroids compared with adherent cells, in concert with an up-regulation of several transcriptional repressors, *i.e.*, *TWIST1/2*, *ZEB2*, and most notably *SNAI1*/Snail. Treatment of EOC spheroids with the TGFβ type I receptor inhibitor, SB-431542, severely reduced Snail expression and potently blocked the endogenous activation of EMT in spheroids. Furthermore, treatment of spheroids with SB-431542 upon re-attachment enhanced the epithelial phenotype of dispersing cells and significantly decreased cell motility and Transwell migration. Spheroid formation was significantly compromised by exposure to SB-431542 that correlated with a reduction in cell viability particularly in combination with carboplatin treatment. Thus, our findings are the first to demonstrate that intact TGFβ signalling is required to control EMT in EOC ascites-derived cell spheroids and it promotes the malignant characteristics of these structures. Next, we sought to investigate the function of Snail, the most robustly-upregulated EMT marker in EOC spheroids, since its role in EOC metastasis particularly in spheroids is largely unknown. Knockdown of *SNAI1* encoding Snail led to a modest reduction in spheroid formation and cell viability, but we observed a significant decrease in the ability of spheroids to migrate due to loss of Snail expression. Further to this, we retrovirally-transduced the Hey ovarian cancer cell line to ectopically express Snail. HeySNAIL cells exhibited a strong EMT phenotype in adherent culture, a result which correlated with significantly enhanced spheroid cell migration, and adhesion and invasion of spheroids to fibronectin and collagen matrices. Furthermore, using an *ex ovo* chick embryo chorioallantoic membrane assay, Snail-expressing EOC spheroids possessed significantly

enhanced tumour-forming potential, invasion, and liver metastasis. These results highlight the therapeutic potential for targeting the Snail/EMT/TGFβ signalling pathway in patients with late-stage ovarian cancer.

Keywords

Ovarian Cancer, EOC, high-grade serous, ascites, spheroids, metastasis, EMT, TGFβ, Snail

Co-Authorship Statement

The data presented in Chapter 2 appeared in the published manuscript "TGFβ signalling regulates epithelial-mesenchymal plasticity in ovarian cancer ascites-derived spheroids." Samah Rafehi, Yudith Ramos Valdes, Monique Bertrand, Jacob McGee, Michel Préfontaine, Akira Sugimoto, Gabriel E. DiMattia, Trevor G. Shepherd. *Endocrine Related Cancer.* 2016. 23(3): 147-59. All experiments were performed and analyzed by S Rafehi. Y Ramos Valdes provided technical assistance. M Bertrand, J McGee, M Prefontaine, and A Sugimoto contributed reagents and materials. S Rafehi and TG Shepherd wrote the paper. The manuscript was edited by S Rafehi, GE DiMattia and TG Shepherd.

In Chapter 3, Y Ramos Valdes performed the anchorage-independent growth assays (Figure S3.4B). All other experiments were performed and analyzed by S Rafehi. M Cecchini helped with histologic data analysis. D Johnston and S Penuela contributed materials and provided technical assistance. The manuscript was written by S Rafehi and edited by GE DiMattia and TG Shepherd.

All other Chapters were written by Samah Rafehi and edited by Dr. Trevor Shepherd.

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List of Abbreviations

AKT	Protein kinase B
B-raf	v-raf murine sarcoma viral oncogene homolog B1
BMP	Bone morphogenic protein
BRCAI1/2	Breast cancer growth suppressor protein1/2
chickCAM	Chick Chorioallantoic Membrane
CDKN2B	Cyclin-dependent kinase inhibitor 2B
Co-Smad	Common mediator Smad
E-cadherin	Epithelial Cadherin
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMT	Epithelial-to-mesenchymal transition
EOC	Epithelial ovarian cancer
EpCAM	Epithelial cell adhesion molecule
ERK	Extracellular signal-regulated kinase
EVI1	Ecotropic virus integration site 1
GSK-3β	Glycogen synthase kinase-3 beta
HDAC1/2	Histone deacetylase 1/2
HGF	Hepatocyte growth factor
HGSC	High-grade serous carcinoma
HIF-1α	Hypoxia-inducible factor-1alpha
I-SMADs	Inhibitory SMADs
JAK/STAT	Janus Kinase/Signal Transducer and Activator of Transcription
JNK	c-Jun N-terminal kinase
K-ras	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
MAPK	Mitogen-activated protein kinase
MET	Mesenchymal-to-epithelial transition
miRNA	micro ribonucleic acid
MIS	Mullerian inhibiting substance
N-cadherin	Neural cadherin
Pak1	P21-activated kinase
PDGF	Platelet-derived growth factor

PI3-kinase	Phosphoinositide 3-kinase
PTEN	Phosphatase and tensin homologue
R-Smad	Receptor-activated Smad
RAS	Retrovirus associated sequence oncogene
Rho GTPase	Ras homologue guanine triphosphotase
SBE	Smad-binding elements
SMAD	Similar to Mothers Against Decapentaplegic
SMURF1/2	Smad ubiquitination regulatory factors1/2
STIC	Serous tubal intraepithelial carcinoma
TβRI/ALK5	TGFβ type I receptor
TβRII	TGFβ type II receptor
TGFβ	Transforming growth factor-beta
TP53	Tumour protein 53
TRAF	Tumour necrosis factor receptor associated factor
TAK1	TGFβ-activated kinase 1
Wnt	Wingless-type mouse mammary tumour virus integration site family member

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Chapter 1

1 INTRODUCTION

1.1 Overview of Chapter 1

This thesis focuses on investigating the plasticity of ovarian cancer cells in terms of their epithelial and mesenchymal states when they aggregate into 3D formations (spheroids) and re-attach onto substratum, and whether this process is dynamically regulated by members of the TGFβ/SMAD signalling pathway. Through my investigations, we believe to have uncovered important aspects of ovarian cancer spheroid formation and metastasis. This chapter begins with a description of ovarian cancer (Section 1.2) specifically focusing on the origins, classification, and mortality associated with this very complex disease. The next section (Section 1.3) focuses on the spheroid as an *in vitro* model of ovarian cancer metastasis and the unique properties that spheroid cells acquire to avoid anoikis and propagate disease. Epithelial-to-mesenchymal transition (EMT) (Section 1.4) and the transforming growth factor beta (TGFβ) (Section 1.5) signalling pathway will be described. Snail/*SNAI1*, a downstream target of TGFβ signalling and one of the main inducers of EMT will also be described (section 1.4.4). The relevance of these topics to ovarian cancer will be highlighted, and my data in the following Chapters (2 and 3) will show that these pathways are important for the formation, survival, and invasive capabilities of ovarian cancer spheroids. The final section of this chapter provides rationale for our research (Section 1.6) and outlines the studies presented in this thesis.

1.2 Ovarian Cancer

1.2.1 Classification

Ovarian cancer may be of germ cell, stromal, or epithelial origin [1]. Epithelial ovarian cancer (EOC), the most common type, is the focus of my thesis. Rather than existing as a single entity, EOC appears to be a group of distinct diseases that can be further classified into the following five histological subtypes: serous, endometrioid, mucinous, clear cell, and undifferentiated carcinomas [2]. These tumours morphologically resemble various mucosal tissues of the female reproductive tract. For example, serous tumours

resemble fallopian tube epithelium, endometrioid tumours resemble endometrial glands, and mucinous tumours resemble endocervical glands or gastrointestinal epithelium [3]. Among these subtypes, high-grade serous carcinoma (HGSC) is the most prevalent and is responsible for the highest fatality rate [4, 5].

A dual classification system of type I and type II tumours has been proposed that incorporates genetic profiling of tumours as well as histology and clinical behavior [6, 7]. Type I tumours include low-grade serous, endometrioid, mucinous as well as clear cell carcinomas, whereas type II carcinomas consist mostly of high-grade serous carcinomas, carcinosarcomas and undifferentiated carcinomas [7, 8]. Low-grade carcinomas are slow growing and generally more indolent, and often confined to the ovary at diagnosis. In contrast, high-grade tumours are clinically aggressive at initial presentation, metastasize early, and are associated with poor clinical outcome [9]. Type I tumours usually present with mutations in K-ras, B-raf, β-catenin, PI3-kinase pathway and/or *PTEN*. In contrast, Type II tumours display high levels of genomic instability and are characterized mainly by *TP53* mutations [7, 8]. The Cancer Genome Atlas identified a high frequency (96%) of high-grade serous tumours to harbor *TP53* mutations. As these genetic alterations occur early in the transformation process, the contribution of these genes is suggested to define the tumourigenic process as well as histological differentiation.

1.2.2 Origins

It had been long accepted that all EOCs are derived from the ovarian surface epithelium (mesothelium) and that subsequent metaplastic changes lead to the development of the various cell types that morphologically resemble the epithelium of the female reproductive tract [10, 11]. Evidence from the past decade suggests that some human HGSCs may originate from secretory epithelial cells of the fallopian tube. Until recently, early cancerous or precancerous lesions in the fallopian tubal fimbria (serous tubal intraepithelial carcinoma or STIC) had been identified in women harboring the *TP53* mutation as well as *BRCA1* or *BRCA2* dysfunction in sporadic pelvic serous carcinoma. On the basis of these initial studies, and a surge of evidence to follow, the fallopian tube has now emerged as an important source for HGSC [12-14].

1.2.3 Treatment and prognosis

Ovarian cancer is called the ‘silent killer’, since there is no effective diagnostic method for detecting these tumours at an early stage and more than 75% of patients have advanced disease at initial diagnosis [15]. Around 20% of ovarian cancers are diagnosed while they are still limited to the ovaries (stage I). At this stage, up to 90% of patients can be cured using currently available therapy. After the disease has metastasized to the pelvic organs (stage II), the abdomen (stage III) or beyond the peritoneal cavity (stage VI), the cure rate decreases substantially [16].

Epithelial ovarian cancer, which constitutes 90% of all ovarian malignancies, is the most lethal disease amongst gynecologic malignancies in the western world [17, 18]. Because of its absence of obvious symptoms and as mentioned above, lack of effective screening tests, the diagnosis of EOC is often made when the disease is at an advanced stage, when tumour has spread beyond the ovaries and disseminated into the peritoneal cavity, resulting in a survival rate of only 20– 30% [19]. Reasons for this high lethality are not only due to the advanced stage at which patients are diagnosed, but also due to the inherent aggressive biology of this cancer. Ovarian cancer differs from hematogenously spreading tumours in that the cells detach from the primary tumour and disseminate throughout the peritoneal cavity, by the flow of peritoneal fluid, to settle onto peritoneal surfaces forming numerous nodules. The degree of peritoneal dissemination correlates with a worse prognosis in patients with advanced-stage ovarian cancer.

The standard therapy for advanced ovarian cancer is multidisciplinary treatment that combines maximum tumour-debulking surgery with taxane (paclitaxel) and platinum (carboplatin) based chemotherapy [20]. Particularly in patients with advanced ovarian cancer, it is extremely important to remove as much of the tumour as possible by appropriate debulking surgery. However, this is extremely difficult because of diffuse tumour spread within the peritoneum. Therefore, almost all patients are treated with subsequent chemotherapy to eradicate the residual microscopic and macroscopic peritoneal metastases. Initially, ovarian cancer is generally sensitive to anticancer drugs, approximately 70% of patients can achieve remission with the standard taxane and

platinum based chemotherapy [21, 22]. Unfortunately, this remission is short lived (lasting only 1-2 years after the initiation of treatment) and relapsing tumours are often resistant to chemotherapy. Patients inevitably succumb to their disease as a consequence of tumour recurrence and metastatic spread [22, 23]. Thus, the long-term prognosis of patients with advanced ovarian cancer is very poor. The biological features of tumours and their sensitivity to anticancer drugs can also depend on the tumour histology, making matters even more complex for treatment [24-26]. Due to the limitations of current standard therapy for ovarian cancer, new treatment strategies to improve the long-term prognosis of patients with this cancer need to be examined.

1.2.4 **Metastasis**

The diffuse nature of ovarian cancer metastasis renders treatments increasingly ineffective. The intraperitoneal metastasis of ovarian cancer is unique among solid tumours, as most of the women with advanced-stage ovarian cancer have diffuse peritoneal spread with no clinically apparent hematogenous metastases [27, 28]. Peritoneal dissemination is the most frequent route of spread and is the most significant prognostic factor in ovarian cancer [29]. Ovarian cancer cells commonly shed from the primary tumour directly into the peritoneal cavity either as multicellular clusters, or as individual tumour cells that can aggregate into multicellular clusters or spheroids (Figure 1.1). This is usually associated with a buildup of malignant pertioneal fluid known as ascites, particularly in advanced high-grade serous carcinomas. Ascites accumulation occurs through the combined effect of lymphatic obstruction by metastatic cancer cells, increased production of by mesothelial cells, and increased vascular permeability [30-32]. Spheroids free floating in ascites survive and subsequently spread and attach to the peritoneum (Figure 1.1) and other intra-abdominal organs including the omentum, diaphragm, and small bowel mesentery [33-35]. All of the organs within the peritoneal cavity are lined with a continuous monolayer of mesothelial cells [36-38]. Localized proteolytic degradation of mesothelial extracellular matrix facilitates the migration of cancer cells allowing them to

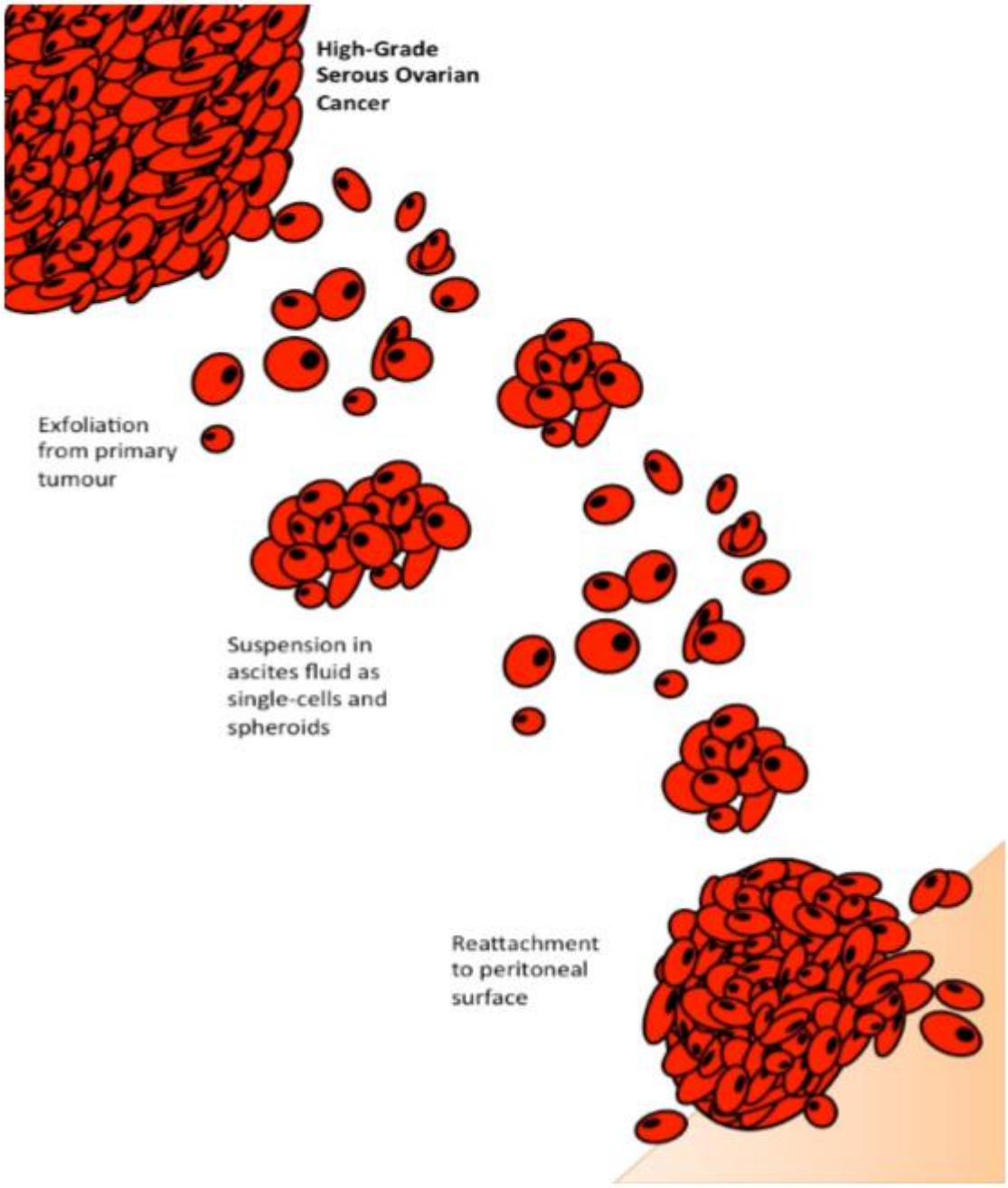

Figure 1.1: Mechanism of high-grade serous ovarian cancer metastasis.

During the process of ovarian cancer metastasis, malignant cells are shed from the primary tumour into the peritoneal cavity. It is here, suspended within ascites fluid, that single-cells and multicellular aggregates (spheroids) disperse throughout the peritoneal cavity. Widespread secondary metastatic lesions are formed when cells re-attach to mesothelial surfaces throughout the peritoneal cavity.

anchor as secondary lesions on pelvic organs and at a later stage, metastasize to distant organs [39]. Hence, processes such as cell-cell, cell-ECM, localized intraperitoneal migration and invasion of the peritoneal lining by spheroids play a dominant role in ovarian cancer progression. The molecular mechanisms underlying such metastasis remain poorly understood, and effective eradication of peritoneal dissemination remains challenging. Therefore, there is an urgent need to develop new treatment modalities, especially targeted molecular therapies, through the study of basic biology underlying peritoneal EOC spread.

1.3 Multicellular spheroids

1.3.1 Spheroids as an *in vitro* model of metastasis

In order to better comprehend tumour cell biology, it is necessary to utilize *in vitro* models that are able to represent *in vivo* tumours as precisely as possible. Most current information about cancer metastasis and invasion is obtained by the study of cells on 2D surfaces. However, monolayer cultures cannot accurately reproduce the behavior of cancer cells within solid tumours. In reality, cells are physically organized in 3D patterns surrounded by ECM and other cell types, and their behavior is affected by exposure to gradients of O_2, glucose, and other nutrients, as well as growth factors; as a result, distinct transcriptional and biochemical regulation ensues. Thus, 3D tumour spheroid systems have been developed and used as an *in vitro* model to recapitulate the *in vivo* tumour microenvironment and study the initial steps of invasion from a primary tumour [40-42]. The 3D organization of cells in spheroids quite faithfully mimics the intercellular interactions and the close communications between cells in solid tumours. Several reports have described the roles of cadherins (Ca^{2+}-dependent transmembrane proteins that bind to each other by homophilic interactions) and integrins (proteins that function as heterodimers of α- and β-subunits and act to link cells to ECM proteins) in the spheroid-forming process [43-46]. It has been documented in glioma, breast, cervix, and prostate carcinoma cells (BMG1, MCF-7, HeLa, and PC3 cell lines) that the acquisition of a 3D structure induces alterations in the cellular microenvironment promoting significant changes in tumour energy metabolism, proliferation rates, transcription factors, oncogene profile, and drug sensitivity compared to conventional 2D cultures [47-51].

Spheroid based assays have been shown to better recapitulate drug resistance than monolayer cultures [52, 53]. In particular, it has been shown that spheroids are more resistant to chemical (antitumour drugs) and physical (ionizing radiation) agents than monolayers and that this resistance is comparable to that found in *in vivo* tumours [54]. The development of slower or non-proliferating (quiescent) and hypoxic (sometimes necrotic) cells in the inner zones of spheroids also more closely resemble those found in tumours. It is therefore advantageous to use 3D spheroid models over 2D culture systems for studying the process of tumour metastasis that could pave the way for better treatment modalities and perhaps even prevention.

1.3.2 Spheroids in ovarian cancer

Multicellular spheroids are valuable tools for the study of ovarian cancer not only because they are a more accurate recapitulation of a solid tumour microenvironment than that provided by monolayer culture, but also because of the unique way ovarian cancer metastasizes. In the initial steps of EOC dissemination, malignant cells detach from the primary tumour directly into the peritoneal space, where single cells do not survive for a very long time if they remain alone and must aggregate to other cells in order to survive and resist apoptosis, or cell death by detachment, better termed as “anoikis” [55]. The aggregation process depends largely on the expression of certain cell-cell interacting molecules, which appear dramatically different in spheroids compared to monolayer growing cells [56, 57].

Protected from the surrounding environmental stresses, these aggregates or spheroids float within the malignant ascites of the peritoneum, facilitating tumour cell invasion into the mesothelium [58]. Iwanicki and colleagues [59] were the first to provide evidence that ovarian cancer spheroids “plow” through the protective layer of mesothelial cells, leading to mesothelial clearance. Others have corroborated such findings [60], and showed that once the cancer cells reach the ECM, they have access to underlying stromal cells, which can be recruited to further support their survival and rapid growth. The secretion of matrix metalloproteinases (MMPs) by EOC spheroids, specifically MMP2 and MMP9, has also been shown to aid in the invasion and degradation of ECM [57, 61].

Furthermore, it's been shown that *in vitro* cultured spheroids are capable of tumourigenesis *in vivo* and display a reduced response to chemotherapeutic drugs when compared to monolayers [62-64]. EOC spheroids have been shown to undergo reduced proliferation and limited drug penetration resulting in decreased susceptibility to chemotherapy [65] and hence, are likely the source of recurrent disease. It is well accepted now that spheroids represent a significant impediment to successful treatment of late stage EOC [33].

Though it is apparent that spheroids may make a significant contribution to intraperitoneal ovarian cancer spread since they are resistant to anoikis, invade mesothelium, and acquire resistance to chemotherapy, much remains to be elucidated regarding their complex biology. Since ovarian cancer mortality can be directly attributed to disseminated peritoneal mestastasis, gaining a better understanding of spheroid biology is crucial in allowing us to identify new treatment opportunities to combat disease spread.

1.4 EMT

1.4.1 Overview of EMT in cancer

The term epithelial-to-mesenchymal transition (EMT) describes a multi-step event during which cells lose numerous epithelial characteristics and gain the properties typical for mesenchymal cells. EMT is an essential morphological conversion that occurs during embryonic development for complex body patterning. Transitions in cell phenotype from epithelial to mesenchymal (EMT) or mesenchymal to epithelial (MET), play a crucial role during developmental and physiologic programs that orchestrate events, such as neural crest formation, palatal fusion, and wound healing [66, 67]. Under the appropriate conditions, epithelial and mesenchymal cells interconvert between these two phenotypes; as such, these phenotypes are not always permanent. It is well accepted that the reactivation of developmental programs can occur during tumourigenesis, and an analogous plasticity of cellular phenotypes occurs in cancers too. In fact, cancer cells in advanced stages become aggressive and invasive due to the pathologic initiation of an EMT. Studies on human and mouse tumours suggest that the same molecular processes that drive developmental EMT are reactivated in the tumour cell to drive tumour progression towards invasive metastatic carcinomas [68].

Cells undergoing these dynamic transitions, whether under physiologic conditions or tumourigenesis, require complex changes in gene expression, cell architecture, and migratory and invasive behavior. Typically, epithelial cells involved in an EMT undergo dissolution of cell-cell junctions (adherens junctions and desmosomes, tight junctions, and gap junctions), lose apical-basolateral polarity, and acquire a mesenchymal phenotype, characterized by actin reorganization and stress fiber formation, migration and invasion [69-72]. The loss of cell-cell adhesion is a prerequisite of EMT. One of the essential molecules for formation and maintenance of the epithelial phenotype is the adhesion molecule E-cadherin (encoded by *CDH1*) which is typically located at cell-cell adherens junctions. E-cadherin is a transmembrane glycoprotein which connects through α- and β-catenin to the actin microfilaments within the cytoplasm, thereby anchoring epithelial cells to each other. Loss of E-cadherin is consistently observed during EMT and is currently regarded as a hallmark of EMT [67, 73]. Other epithelial markers that may be downregulated during this process include ZO-1, claudins, occludin, and cytokeratins. At the same time, up regulation of transcription factors such as Snail, Slug, Twist1/2, Zeb1/2, as well as mesenchymal markers such as vimentin and fibronectin, can lead to the acquisition of cell motility and invasive properties. These changes may further endow cancer cells with the ability to secrete proteases that degrade the extracellular matrix and migrate to form distant metastases [67, 74-76]. Researchers have used many different markers to track pathological EMT, the most commonly reported changes are summarized in Table 1.1. Additionally, cytoskeletal elements are reorganized and the peripheral actin cytoskeleton is replaced by stress fibers, whereas cytokeratin intermediate filaments are replaced by vimentin. Together with gene expression changes, these morphological changes transition the cell from a compact, cuboidal (epithelial) shape to a spindle-like, fibroblastic (mesenchymal) phenotype. Such transitions render cancer cells to further become motile by loosening cell-cell contacts and cell polarity [77].

Such features of EMT have been observed in a wide variety of different cancer models, including ovarian cancer [78-81]. Numerous EMT inducers characterized in cell culture systems can initiate metastatic spread in animal models, and in fact correlate with poor prognosis in human cancer [82, 83]. For instance, human hepatocellular carcinoma

Table 1.1: Classical markers of EMT

Cell-surface proteins	
N-cadherin	↑
OB-cadherin	↑
α5β1 integrin	↑
αVβ6 integrin	↑
Syndecan-1	↑
E-cadherin	↓
Z0-1	↓
Claudin	↓
Occluidin	↓
Cytoskeletal markers	
FSP1	↑
α-SMA	↑
Vimentin	↑
β-Catenin	↓
Cytokeratin	↓
ECM proteins	
α1 (I) collagen	↑
α1 (II) collagen	↑
α1 (IV) collagen	↓
Laminin 1	↓
Transcription factors	
Snail1 (Snail)	↑
Snail2 (Slug)	↑
ZEB1	↑
ZEB2	↑
Twist1	↑
Twist2	↑

(Agiostratidov et al. 2007, Hugo et al. 2007, Venkov et al. 2007, Zeisberg et al. 2009, Kallergi et al. 2011, Quaggin et al. 2011)

(HCC) cell lines harboring both Snail and Twist showed a higher migratory and invasive ability *in vitro*, and co-expression of Snail and Twist indicated the worst outcome in *in vivo* mouse models [84]. Furthermore, Snail or Twist overexpression (associated with downregulated E-cadherin and β-catenin expression) was shown in recurrent versus primary HCCs specimens, and has been associated with worse progression-free and overall survival in patients [84]. Furthermore, invasive and metastatic properties of mouse and human tumour cell lines derived from tissues such as breast, colon, liver, skin and oral mucosa have shown a direct correlation with Snail expression. Snail has been shown to be expressed at the invasive front of skin tumours induced by chemical carcinogenesis in the mouse [85-89]. Recent studies have shown that EMT not only endows cancer cells with motility to detach from neighboring cells, but also promotes anoikis resistance in cancer cells in anchorage-independent circumstances [90, 91]. Slug-mediated EMT was found to promote cell motility and contribute to the acquisition of anoikis resistance in human adenoid cystic carcinoma cells [92].

Emerging evidence also suggests that EMT may be associated with the acquisition of chemotherapeutic resistance. Colon carcinoma cell lines resistant to oxaliplatin were shown to exhibit a mesenchymal morphology and express several markers of EMT [93]. Suppression of Twist in the lung carcinoma cell line A549 resulted in increased chemosensitivity to cisplatin [94]. Snail expression has been shown to confer resistance to paclitaxel, adriamycin, and radiation by antagonizing p53-mediated apoptosis [95, 96]. Increased expression of Zeb1 and decreased expression of E-cadherin have been associated with drug resistant pancreatic cell lines, and reduction of Zeb1 expression has been implicated in increased drug sensitivity [97]. Twist and one of its target genes were shown to be elevated in a subset of MCF7 or MDA-MB434 breast cancer cells selected for their invasive properties, and, having undergone EMT, they were also resistant to paclitaxel [98]. Moreover, the depletion of Twist can partially reverse multidrug resistance in breast cancer cells [99].

Other studies have also shown EMT induced by EGFR signalling to be linked to tamoxifen resistance and increased invasiveness of MCF7 breast cancer cells [100, 101]. Evidence from several other groups have further demonstrated that the induction of EMT

in breast cancer cells results in the acquisition of stem-like properties , including the ability to form mammospheres in culture and to resist the cytotoxic effects of chemotherapy [102, 103]. This idea that EMT can induce stem cell-like properties in cancer cells is largely growing and continues to be tested in a variety of different cancers, including EOC. Altogether, the expression of EMT signalling pathways has been associated with cancer progression, relapse and poor prognosis in various epithelial cancers, including breast, pancreas, colon, prostate, and ovarian cancer [104-106]. The EMT program seems to give carcinoma cells the phenotypic traits necessary for invasion and metastasis as well as for resistance to cancer therapies. Targeting the molecular activators of EMT and downstream transcriptional regulators of EMT are thus hypothesized to be a potentially effective way of treating cancer patients.

1.4.2 Regulation of EMT

EMT is controlled by a network of signalling and transcriptional events. Not surprisingly, many of the same signalling pathways and transcription factors important to physiologic instances of EMT are also activated during pathologic EMT. A variety of cytokines and growth factors are induced during EMT, which further stimulate the EMT process and promote cell migration and invasion; these factors include transforming growth factor β (TGFβ), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), Wnt, Notch ligands, and others [107-109]. Hypoxia can also induce EMT in tumours through the up regulation of hypoxia-inducible factor-1α (HIFα), which affects transcription of target genes [110]. Although several growth factors participate in EMT, TGFβ is the prototypical inducer of EMT, and is the most studied [111].

The process of EMT is further regulated by several transcriptional suppressor families. These ‘master regulators’ of EMT include the zinc finger proteins Snail/*SNAI1* and Slug/*SNAI2*, the two-handed zinc finger family factors Zeb1/*ZEB1* and Zeb2/*ZEB2*, and the basic helix-loop-helix factors Twist1/*TWIST1* and Twist2/*TWIST2* [112-114]. All of these transcription factors have been shown to be involved in disrupting epithelial homeostasis by destabilizing cell-cell contacts, a critical step in EMT. For example, the

SNAI and *ZEB* family of transcription factors can bind directly to the E-cadherin promoter to repress gene transcription [115]. Previous studies demonstrated that this is mediated by specific binding to E-box sequences within the *CDH1* promoter [116]. Snail is the first discovered and most important transcriptional repressor of *CDH1*. Snail induces repressive histone modifications at the *CDH1* promoter through the recruitment of co-repressors and different proteins, such as histone deacetylase 1 (HDAC1) and HDAC2 [117]. Activation of the TGFβ pathway itself can recruit Snail [118], Zeb [119], or Twist [120] to repress epithelial genes. TGFβ/Smad pathway can transcriptionally induce Snail expression by directly binding to the *SNAI1* promoter.

During the progression of EMT, it is believed that Snail will be the first factor to become active to initiate the transition whereas Slug would be expressed in later stages to allow the cells to retain their mesenchymal characteristics [121]. Snail has been shown to be sufficient to induce EMT and the expression of EMT associated genes, suggesting that Snail may act as the switch to promote the EMT program in epithelial cells [112]. For instance, in tumours that express Snail, *ZEB* gene activation frequently occurs upon *SNAI1* gene transcription [122]. Some studies have demonstrated that ectopic expression of Snail alone can promote an EMT in epithelial tumour cell lines [88, 89]. Snail not only represses *CDH1* expression through direct binding to E-boxes; other genes that may also be negatively regulated by Snail include cytokeratin 18, desmoplakin, MUC1, claudin, and occludin, whereas the expression of matrix metalloproteinases, vimentin, fibronectin, and Rho GTPases can be induced by Snail during EMT [112, 123, 124]. The transcriptional repressor activity of Snail is modulated by its expression level and intracellular localization. For example, as previously mentioned, certain growth factors/signalling pathways (such as TGFβ) can modulate snail expression. In terms of localization, p21-activated kinase (Pak1), a serine/threonine protein kinase, can regulate Snail through phosphorylation at Ser246, causing Snail to accumulate in the nucleus where it is most stable and resulting in enhanced transcriptional activity. Cytoplasmic Snail, on the other hand, has a very short half-life as it is targeted for ubiquitin-mediated proteasome degradation by glycogen synthase kinase-3β (GSK-3β) induced phosphorylation [125].

Several recent studies have also recognized the role of non-coding RNAs in activating the EMT program in cancers [126]. In this context, the role of miR-200 family has been well characterized in regulating EMT [127]. Expression of the miR-200 family is strongly associated with epithelial differentiation, and a reciprocal feedback loop between the miR-200 family and the ZEB family of transcription factors tightly controls both EMT and MET [127, 128]. The miR-200 family members, considered to be tumour suppressive miRNAs, have been shown to be downregulated in a variety of different cancers including bladder, gastric, head and neck, and ovarian cancer [129-133]. Other microRNAs considered to be oncogenic have also been indicated in the regulation of EMT.

1.4.3 EMT in ovarian cancer

Among the panel of solid tumours that EMT has been implicated to play crucial roles, EOC is probably among the least understood and studied. The clinical relevance of EMT in ovarian cancer development and progression is not fully understood, in part, owing to conflicting results with EMT marker gene expression patterns and clinical outcomes. For example, the loss of E-cadherin may correlate with poor survival [134, 135], but in an additional large-scale study the opposite correlation in high-grade EOC was observed [136]. EOC represents a heterogeneous disease entity with unique biology [137]. Unlike other carcinomas, many EOC tumours have been shown to co-express epithelial and mesenchymal determinants to begin with [138, 139]. Nevertheless, recently, there has been an increase in findings implicating EMT in ovarian carcinoma and in promoting EOC aggressiveness [139-141]. It has been suggested that primary EOC may undergo an EMT-like process during localized invasion in the peritoneum and retain mesenchymal features in advanced tumours [142].

The shedding of cancerous cells from the primary tumour into the peritoneal cavity is the first step of the metastatic spread. This peritoneal spread of ovarian cancer to surrounding organs within the abdomen relies on the movement of cancer cells, which can be regulated in an autocrine manner, or in a paracrine manner by growth factors and cytokines present in the surrounding microenvironment or peritoneal fluid [143]. In this context, the contribution of EMT by endogenous or exogenous growth factors is vital to

initiate and sustain abdominal cell motility of tumour cells. In fact, similar to other cancers, there are studies in the literature indicating that signalling pathways such as the TGFβ, EGF, JAK/STAT, MAPK, ERK, AKT and other cytokines present in ascites fluid can either trigger or stabilize EMT in ovarian cancer cells, contributing to a more motile and aggressive phenotype [144-149]. Thus, more recent studies support the hypothesis that induction of the EMT program is important for ovarian cancer progression and metastasis [150], however, the exact underlying mechanisms of EMT-mediated EOC metastasis needs further research, especially in the context of spheroid cell dissemination.

As with other cancers, emerging evidence suggests that EMT plays a crucial role in the progression of EOC not only by increasing cancer cell motility, but also by inducing chemo-resistance, and cancer stem cell characteristics [151, 152]. Ovarian carcinoma cells that are resistant to paclitaxel have been shown to exhibit relatively high expression of Twist and Snail and loss of an epithelial phenotype. These paclitaxel-resistant EOC cells further displayed enhanced motile and invasive behavior *in vitro* and increased formation of peritoneal metastasis in a mouse model compared to their paclitaxel-sensitive counterparts [153]. Exposure of clinical specimens from patients diagnosed with late-stage ovarian cancer to cisplatin, as well as the OVCA433 cell line, showed reduced expression of *CDH1* and increased *SNAI1*, *SNAI2*, *TWIST*, and *VIM* (vimentin) mRNA levels, as well as increased expression of cell surface stem cell markers [154]. In another study using EOC cell lines, upregulation of Snail and Slug have been correlated with resistance to radiation and paclitaxel and shown to directly participate in pro-survival signalling and acquisition of stem-like characteristics [96]. Another study demonstrated, through the use the A2780 ovarian cancer cell line, and its cisplatin-resistant daughter line, A2780cis, that EMT related genes directly contribute to cisplatin-resistance in ovarian cancer. Furthermore, knockdown of *SNAI1* and *SNAI2* in A2780cis showed reversal of the EMT phenotype and restoration of cisplatin sensitivity [155]. The same study assessed a cohort of primary ovarian tumours and their resulting gene expression data mirrored the finding that an EMT-like pathway is activated in resistant tumours relative to sensitive tumours.

Thus, it is not surprising that the induction of EMT in EOC is associated with poor prognosis [156]. In a review summarizing gene expression array analyses, EMT

(sometimes associated with TGF-β signalling) was shown to be a main driver of poor survival in EOC patients [157]. Authors of this review showed that amongst 154 genes highly up-regulated or down-regulated in aggressive EOC, 108 (70.1%) genes were associated with EMT. Another study showed that the expression of E-cadherin transcriptional repressors increased with advanced malignancy, and the prognosis of ovarian carcinoma patients positive for Snail expression was poorer than that of negative patients [158]. In a series of 143 ovarian cancer patients, a recent study showed that high expression of *ZEB2* mRNA significantly correlated with a poor prognosis in terms of both overall survival and progression-free survival [159]. Pathways or expression profiling signatures enriched in EMT genes have been identified to distinguish subgroups of EOC with worse clinical survival outcomes [160]. Therefore, targeting EMT in EOC might be a feasible and effective therapeutic option.

1.4.4 Snail/*SNAI1* in ovarian cancer

The zinc finger factor Snail belongs to the Snail superfamily of transcriptional repressors [123, 124], in which other members associated with EMT are found, such as Slug. Given that Snail is an important initiator of EMT [121], it is not surprising that this transcription factor is linked to the aggressive behavior of ovarian cancer cells. Snail is perhaps one of the most important transcription factors, not only because it acts primarily as a key inducer of EMT, but also because it is strongly correlated with tumour recurrence, cell survival, immune regulation and stem cell biology [125]. However, Snail's role in the pathobiology of EOC is understudied in 3D cell culture systems.

In 2D experimental models of EOC, Snail was shown to mediate E-cadherin downregulation induced by hypoxia [161], promote tumour growth and metastasis [162], induce EMT initiated by p70 S6 kinase and BMP4 [163, 164], and mediate invasion [165]. Ectopically expressed *SNAI1* or *SNAI2* induced EMT in serous adenocarcinoma SKOV3 cells and enhanced motility, invasiveness and tumourigenicity. In this model, Snail was shown to suppress expression of adherens and tight junction components, while Slug further suppressed expression of desmosomes [165]. In an ovarian cancer clear cell carcinoma cell line (ES-2), *SNAI1* shRNA-expression reduced invasion and MMP-2

activity by these cells [166]. Another study deleted Snail in a serous adenocarcinoma cell line (RMG1) using the CRISPR/Cas9 system and showed that *SNAI1* regulates actin cytoskeletal structure and cell migration, as well as cell-substrate adhesion [167]. Furthermore, silencing of Pak1 (which regulates nuclear localization of Snail) in ES-2 and high-grade serous OVCAR3 cells, using dominant negative (DN) clones, showed that the latter had reduced attachment to ECM proteins (laminin, fibronectin, collagen I and IV), invasion, and MMP-2 activity compared to constitutively active (CA) and wild-type ES-2 cells and OVCAR3 cells. DN Pak1 ES-2 cells additionally had reduced binding to LP9 cells, a normal human mesothelial cell line generated from benign mesothelium [166]. Snail up-regulation has also been linked to the increased invasiveness in EOC cells induced by 17beta-Estradiol (E2) [168]. Depletion of endogenous Snail using siRNA attenuated E2 mediated decreases in E-cadherin expression, migration and invasion in EOC cells [169]. All of these studies help us gain an appreciation for the Snail's role in 2D cell culture systems, however, very little is known about the functional role of Snail in EOC spheroids.

Snail expression and its clinical role has been the subject of several studies. One study showed Snail expression to be predominant in serous and endometrioid primary adenocarcinomas by immunohistochemistry, and found correlations between Snail expression, chemoresistance, and poor prognosis [170]. Other clinical studies, however, have reported that Snail represses E-cadherin in primary carcinomas and its expression is increased in metastases; these observations were associated with shorter overall survival [171]. Others have also found significantly higher Snail expression in solid metastases, as well as primary carcinomas from patients diagnosed with late-stage ovarian cancer (III and IV) compared with early-stage ovarian cancer, and Snail was observed to be more often localized to the nucleus in advanced-stage compared to early-stage cancers [162]. Another group reported similar findings where expression levels of Snail, fibronectin and N-cadherin in stage III and IV EOC tissues were higher than those in stage I and II, whereas expression of E-cadherin and β-catenin decreased with advanced stage of disease [172]. High Snail expression in primary carcinomas has been associated with greater risk of death [173]. Nuclear expression of Snail in EOC tumours was further shown to be significantly higher in patients with peritoneal dissemination than in those without, associating Snail with both progression-free survival and overall survival in ovarian cancer patients [174].

Taken together, along with evidence that Snail contributes to multiple aspects of therapeutic resistance [96, 155], it is unquestionably crucial to investigate the functional role of *SNAI1* in the progression of EOC, especially in the context of the spheroid, that could perhaps pave the way for better therapeutic strategies in the treatment of this disease.

1.5 TGFβ signalling

1.5.1 Overview

Transforming growth factor beta (TGFβ) ligands are members of the TGFβ superfamily of signaling molecules, a family which is comprised of more than 30 closely related proteins including bone morphogenetic proteins (BMPs), activins, inhibins and nodal [175]. TGFβ is a multipotent cytokine that is involved in many diverse cellular processes such as cell growth (inhibition/proliferation), differentiation, apoptosis, migration, invasion, EMT, extracellular matrix (ECM) remodeling, immune-suppression, and embryogenesis in mammals [176]. The complicated role of TGFβ in mediating these cellular activities depends on cell types, growth environment, concentration of ligands, and the presence of other growth factors [177-179]. Under normal circumstances, dynamic regulation of TGFβs is important during embryonic development and for maintaining adult tissue homeostasis. However, in disease states (e.g., cancer, fibrosis, and inflammation) excessive production of TGFβ drives disease progression by modulating cell growth, migration or phenotype. Studying the TGFβ signalling pathway has therefore been crucial in unravelling many of the key steps in cancer metastasis. This section will discuss TGFβ signal transduction, how this pathway is regulated as well as its role in various cancers, including ovarian cancer.

1.5.2 Pathway activation

In humans, three isoforms of TGFβ, which share 75% amino acid sequence homology (TGFβ1, TGFβ2 and TGFβ3) have been described [180, 181]. TGFβ1 (the prototypic form) is the most abundant in most tissues. TGFβ1 and TGFβ3 isotypes are expressed in adult tissues, whereas TGFβ2 expression occurs mainly during development and differentiation [175, 182, 183]. TGFβ ligands are secreted from the cell as homodimers

in their latent precursor form, which cannot bind to its receptor until proteolytically cleaved to their active form.

The canonical TGFβ signalling pathway is activated when mature TGFβ homodimers bind either directly to TGFβ type II receptor (TβRII) or indirectly by binding to β glycan (TβRIII) which then presents the TGFβ homodimers to TβRII. Activated TβRII in turn recruits and activates TGFβ type I receptor (TβRI/ALK5). This active receptor complex composed of two type I and two type II receptors phosphorylates and activates the receptor-activated Smads (R-Smad), Smad2 and Smad3 [181, 184]. Activated R-Smads then form complexes with the common mediator Smad (co-Smad): Smad 4. This heteromeric Smad complex then translocates to the nucleus and, along with other DNA binding cofactors, mediates gene transcription by high affinity binding to Smad-binding elements (SBE) in the promoters of its target genes [185] (Figure 1.2). The output of a TGFβ response is highly contextual throughout development, across different tissues, and in cancer [111].

1.5.3 Pathway attenuation

The inhibitory SMADs (I-SMADs: such as SMAD7) negatively regulate TGFβ signalling by competing with R-SMADs for type I receptor or inhibiting the R-SMAD - co-SMAD complex [186, 187]. I-SMADs can also promote proteasomal degradation of R-SMADs and co-SMADs through complex formation with ubiquitin ligases (Smurf1/2) [188-190]. I-SMADs may also antagonize R-SMAD activation by recruiting specific phosphatases to the activated receptor complex, targeting it for dephosphorylation. In addition to being regulated by phosphorylation and ubiquitination, the TGFβ signalling pathway can also be regulated by transcriptional co-repressors (i.e. SnoN/SkiL and EVI1) [191, 192].

1.5.4 Smad-independent signalling

TGFβ signalling is even more complicated. The TGFβ-TβRI-TβRII complex can also signal through mechanisms independent of Smad activation. Activation of non-

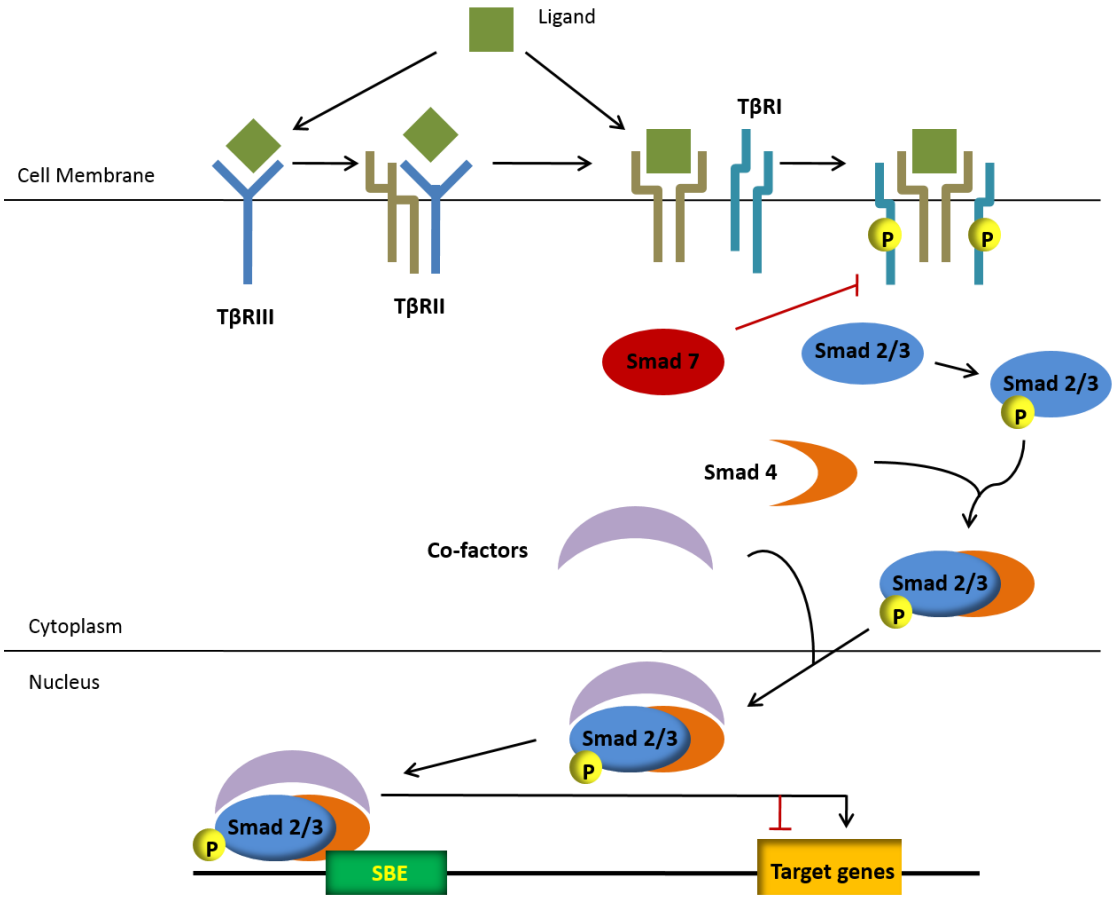

Figure 1.2: Canonical TGFβ signalling pathway.

The canonical TGFβ signalling pathway is activated when active TGFβ binds either directly to TGFβ type II receptor (TβRII) or indirectly by binding to β glycan (TβRIII) which then presents the TGFβ to TβRII. Activated TβRII then recruits and activates the type I receptor (TβRI/ALK5). This active receptor complex composed of two type I and two type II receptors phosphorylates and activates the receptor-activated Smads (R-Smad), Smad2 and Smad3. Activated R-Smads then form complexes with the co-Smad: Smad 4. This heteromeric Smad complex then translocates to the nucleus and mediates gene transcription by binding to Smad-binding elements (SBE) in the promoters of its target genes.

canonical signalling pathways may include the PI3 kinase, ERK/MAPK, TRAF/TAK1/JNK and RhoA/ROCK pathways [191-193]. Moreover, TGFβ signalling output can be strongly influenced by intersecting signalling pathways, such as Ras, Notch and Wnt, and by crosstalk between other ligands, receptors, and SMADs of the TGF-β superfamily [194]. The crosstalk between TGFβ signalling and other signalling pathways is highly context-dependent and could have important implications in cancer [195].

1.5.5 TGFβ signalling in cancer

Many advanced tumours produce excessive amounts of TGFβ which, in normal epithelial cells, is a potent growth inhibitor and plays an important function in normal tissue homeostasis [181, 196, 197]. However, in oncogenically activated cells, the homeostatic action of TGFβ is often diverted along alternative pathways. The current consensus is that TGFβ signalling has a dual role in cancer: while TGFβ signalling elicits protective or tumour suppressive effects during the early growth-sensitive stages of tumourigenesis [197-210], later in tumour development (when tumour cells become refractory to TGFβ-mediated growth inhibition) the TGFβ pathway is utilized for its tumour progressing effects [211]. During the early stages of epithelial tumourigenesis, TGFβ inhibits tumour development and growth by inducing cell cycle arrest, senescence, and apoptosis; this aids in maintaining cellular homeostasis critical for the prevention of continuous cell proliferation and thus tumour formation [191]. At late stages of malignancy, TGFβ overload drives malignant progression and metastasis through three broad biological effects: dampening of immune surveillance [212]; indirect facilitation of tumour cell proliferation (via its effects on the microenvironment - stromal fibroblasts, ECM, and angiogenesis - that in turn modulate the tumour cell) [111, 213, 214]; and cell induction of EMT, which will be the focus of my thesis [215].

TGFβ1, specifically, is secreted in abundance in many advanced human and murine tumours [216-221]. Overexpression of this ligand has been shown to drive metastasis in mouse mammary and skin tumour models [200, 203, 222, 223], melanoma, prostate cancer, colon cancer, as well as many other tumour types [213, 224, 225]. TGFβ in tumours may be produced by cancer cells or by stromal components. In fact, presence of TGFβ in the

tumour microenvironment has been shown to play a prominent role in cancer metastasis. The most vivid example is the bone, with it being a rich store of multiple growth factors including TGFβ, and a common site of dissemination for both breast and prostate cancers [226-228]. Enhanced TGFβ signalling leading to pathogenesis could be mediated in many other ways. For example, one study identified USP15 (a deubiquitinating enzyme) to correlate with high TGFβ activity by binding to the SMAD7-SMURF2 complex, thereby stabilizing TβRI. The USP15 gene was found amplified in glioblastoma, breast and ovarian cancer [229] and conferred poor prognosis in individuals with glioblastoma. Findings from other studies have also identified the TGFβ pathway to be highly active in high-grade glioma, and elevated TGFβ activity further confers poor prognosis in individuals with glioma [230, 231]. The prognostic significance of TGFβ ligands and downstream signalling mediators has also been investigated in several other cancer studies. For example, advanced stages of breast cancer have been associated with elevated TGFβ1 serum levels [232], and high tissue levels of TGFβ1 has been associated with increased lymph node metastasis and an unfavorable prognosis in breast cancer patients [233, 234].

TGFβ has long been known to be a major inducer of EMT particularly in heart formation and palate fusion in mice, as well as in some mammary cell lines, and in mouse models of skin carcinogenesis [215, 235]. In human cancers, TGFβ can promote metastasis via the induction of EMT. Characteristics of EMT have been observed in pathology sections of human cancers, particularly at the invasion front, a location that is rich in stromal TGFβ and other cytokines that may cooperate in the induction of EMT [236]. TGFβ-dependent EMT in cancer cells is mediated, in part, by the ability of TGFβ to alter cell junctions [237-239]. Mouse mammary epithelial cells treated with TGFβ were shown to downregulate the expression of the epithelial markers E-cadherin, ZO-1, desmoplakins I/II and increase the expression of the mesenchymal marker fibronectin [237]. TGFβ-induced EMT has been observed in A549 pulmonary adenocarcinoma cells as well as other cancer cell lines [240, 241].

In addition to its role in enhancing invasion and migration, as alluded to earlier, EMT inducing factors such as TGFβ and its downstream targets (Twist and Snail) may also promote the expression of stem-like cell surface markers [103]. Earlier work has shown

that breast cancer stem cells, identified by the CD44hi/CD24lo marker, overexpress components of the TGFβ pathway. When these cells were treated with a TβRI kinase inhibitor, these stem cells shed their mesenchymal traits to take on a more epithelial phenotype [242].

1.5.6 TGFβ signalling in ovarian cancer

In many cancers including head and neck, breast, lung, esophageal, gastric, colon and pancreatic cancers, resistance to TGFβ's antiproliferative effects has been linked to pathway mutations such as TGFβ receptor I or II (TβRI or TβRII) or Smad 2 and 4 mutations [243-247]; however, in ovarian cancer, TGFβ receptor and Smad mutations are infrequent events [248-251]. In the few cases where receptor and Smad mutations have been described, it is unknown whether these mutations alter protein function [248, 252, 253]. Unlike pancreatic cancer in which ~50% of *SMAD4* is mutated [191], reports of the presence of SMAD4 variants in ovarian cancers are lacking [254]. Polymorphisms in SMADs 2, 6, 7, have been found, however not associated with any amino acid change and therefore unlikely to be significant in the development of ovarian cancer [248, 249]. Results from two separate studies have shown TβRII is not a major mutation target in ovarian cancer [255, 256], whereas another group found that TβRII mutations were significantly associated with clear cell carcinomas [257]. Other groups have shown that, although ovarian carcinomas are resistant to the antimitogenic effects of TGFβ, the TGFβ/Smad pathway remains intact and functional in ovarian cancer cells [258-260]. Since the core pathway components are rarely lost or mutated in ovarian cancer, these results suggest that disruption of TGFβ signalling in ovarian cancer occurs by other mechanisms.

Although TGFβ plays an important role in normal ovarian physiology, there appears to be discrepancies in the literature regarding its role in ovarian cancer progression. For example, some studies show the type II receptor and SMAD4 to be downregulated in ovarian cancer [261]; and negative regulators of TGFβ signalling (such as SMAD7) are overexpressed [259, 262], suggesting that suppression of TGFβ signalling is crucial for ovarian carcinogenesis. However, other studies show that overexpression of TGFβ signalling components, such as SMAD3, is associated with increased metastatic potential

and poor prognosis in ovarian cancer [263]. As stated above, TGFβ signalling functions as a double-edged sword in cancer development, a tumour suppressor in early tumourigenesis but a tumour enhancer in advanced stage cancer. How TGFβ plays a certain role in one cell but a different role in its malignant counterpart is not fully understood. It is likely that ovarian cancer cells derive a selective advantage by keeping a functional tumour-promoting TGFβ signalling pathway while repressing TGFβ antiproliferative responses.

As such, TGFβ1 is specifically thought to play a role in the pathobiological progression of ovarian cancer because this cytokine is overexpressed in cancer tissue, plasma, and peritoneal fluid of ovarian cancer patients [263-265]. Moreover, all three TGFβ ligands have been found to be markedly elevated in ovarian cancer cells [266] and primary ovarian cancer specimens [267]; and mRNA levels of both TGFβ1 and TGFβ3 have been shown to be increased in recurrent ovarian cancers [268]. Quantitative immunohistochemical analysis in another report showed increased TGFβ1 and TGFβ2 expression in both primary and metastatic tumour specimens compared to normal ovarian tissue [269]. Increased expression of TGFβ has been shown to be associated with peritoneal metastasis, expression of VEGF, and microvessel density and thus correlated with poor patient survival [270]. Higher Smad3 expression in ovarian carcinoma has also been shown to be correlated with poor survival [263, 271]. While some studies support a positive association between TGFβ1 expression and prognosis in EOC [270], other studies have found no association [272]. Nevertheless, one study that analyzed the publicly available microarray dataset (GSE2109) found the TGFβ signalling pathway to be activated in omental metastases as compared to primary sites of disease [273]. Omental metastasis is the most frequent site of metastasis and an important prognostic factor in advanced ovarian cancer. Through immunohistochemical analysis, authors of this study found both TβRII and phosphorylated SMAD2 to be increased in omental metastases as compared to primary disease sites [273].

Moreover, TGFβ signalling pathway likely plays an important role as a marker or mediator of chemoresistance in advanced serous ovarian cancer [274]. TGFβ1 mRNA expression was found to be significantly lower in tumours with high sensitivity to paclitaxel and carboplatin than in those with low sensitivity; and those patients whose tumours

showed low expression tended to have a better prognosis than those whose tumours showed high TGFβ1 mRNA expression [275]. Another study found that although the serum level of TGFβ1 had no diagnostic and prognostic role in EOC patients, it was associated with sensitivity to standard chemotherapy. Elevated serum TGFβ1 level was found to be associated with metastatic disease; and chemotherapy unresponsive patients had higher serum TGFβ1 levels compared with responsive ones [276]. Furthermore, a recent pathway analysis of data from nine gene profiling array studies, in which ovarian carcinoma resistance to platinum-based chemotherapy was studied, implicated the TGFβ pathway (which affected both EMT and stemness) as one of the main drivers of chemoresistance in ovarian carcinoma [277].

There is growing evidence in the literature to support that TGFβ signalling may enhance the metastatic potential of ovarian cancer through mediating epithelial to mesenchymal plasticity. However, most of these studies are limited to 2D culture systems. For example, TGFβ1 has been shown to elevate matrix metalloproteinase secretion and invasion through Matrigel in ovarian cancer cells [278, 279]. All three TGFβ isoforms induced secretion of MMPs, loss of cell-cell junctions, down-regulation of E-cadherin, up regulation of N-cadherin, and acquisition of a fibroblastoid phenotype in the OVCA429 cell line [263]. A subsequent study corroborated these findings and showed that TGFβ treatment of OVCA429 cells induced the expression of Snail and Slug, repressed the expression of E-cadherin, and increased the production of MMP2. The EMT phenotype and invasiveness of these cells was further augmented by additional treatment with EGF [280]. Other reports have demonstrated that TGFβ treatment of the SKOV3 ovarian cancer cell line stimulated re-organization of the actin cytoskeleton triggering stress fibers and cellular protrusion formation [78], and decreased the expression of E-cadherin while increasing N-cadherin and expression of vimentin [281]. TGFβ target genes, such as Id-1, was shown to mediate a motile and invasive EMT phenotype in SKOV3 and 3AO ovarian cancer cells as knockdown of Id-1 inhibited these changes [281]. Another study reported that TGFβ1 induces EMT in two ovarian adenosarcoma cell lines, derived from a rare variant of a mixed-Mullerian tumour [282].

One study proposed a unique mechanism by which the TGFβ signalling pathway played an active role in high-grade serous EOC progression. They identified miR-181a as an inducer of TGFβ-mediated EMT, via suppression of Smad7 [283]. Data from a large-scale analysis of numerous high-grade serous ovarian cancer samples suggests that acquisition of invasiveness in ovarian cancer cells is accompanied by the emergence of a mesenchymal subtype of tumours with a TGFβ gene signature that is associated with poor patient outcome [284]. Although some studies have uncovered an association between EOC outcome and TGFβ-mediated EMT, much remains to be elucidated about how this ligand contributes to spheroid-mediated ovarian cancer progression, particularly in the regulation of an EMT.

1.6 Scope of Thesis

The presence of peritoneal metastases in EOC, mainly mediated by spheroid formation and dissemination, is a common and adverse event associated with poor prognosis. Cells from the primary tumour shed into the peritoneal cavity and must survive under non-adherent conditions until they reach the serosal surface of various abdominal organs, upon which they are able to reattach and form secondary metastatic lesions. Our lab has developed an *in vitro* model system to mimic suspension-induced spheroid formation and re-implantation to an adherent substratum. Herein, we provide the first report indicating that spheroid formation alone has the capacity to drive EMT in patient ascites-derived EOC cells, and this is regulated by endogenous TGFβ signalling (Chapter 2). We continue to investigate the role of Snail/*SNAI1* downstream TGFβ signalling in EOC spheroid formation and metastasis (Chapter 3) as we observed this protein to be robustly upregulated during spheroid formation, and we hypothesized this to be a crucial factor in the acquisition of an aggressive phenotype in EOC.

Given the unique way EOC metastasizes, it is particularly important to understand the biology of these more aggressive aggregates in order to prevent or hamper formation of deadly peritoneal metastasis. However, there remains a paucity of literature that explores basic biological phenomena and altered signalling pathways in EOC spheroids, as most studies have frequently employed 2D culture systems for the study of tumour migration

and invasion. As such, our studies have all been performed in a biologically-relevant 3D model system, which reveal some of the complex molecular mechanisms that mediate EOC invasion and metastasis. Our findings may therefore shed light into the identification of novel targeted therapies for the management of EOC metastasis.

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Chapter 2

2 TGFβ SIGNALLING REGULATES EPITHELIAL-MESENCHYMAL PLASTICITY IN OVARIAN CANCER ASCITES-DERIVED SPHEROIDS

2.1 Introduction

Epithelial ovarian cancer (EOC) is the most lethal of the gynaecologic malignancies in women [1, 2]. Early detection significantly enhances survival; however, more than 75% of patients present at first diagnosis with advanced stage disease when the tumour has already metastasized beyond the primary site [3]. EOC is unique among carcinomas in that it very rarely leads to blood-borne metastasis; rather, malignant cells are shed from the primary tumour directly into the peritoneal cavity. Malignant cells aggregate and survive in the peritoneal fluid, often in the form of ascites, as spheroid-like structures. These spheroids then attack and invade the peritoneum, seed metastatic tumour growth, and are resistant to standard chemotherapeutics. Extensive seeding of these spheroids are frequently observed on the uterus, sigmoid colon and omentum in advanced-stage and recurrent disease [4, 5]. Thus, a better understanding of EOC spheroid biology may elucidate important mechanisms involved in the metastasis of this complex disease.

It has been proposed that EOC cells are able to switch between epithelial and mesenchymal states during metastasis [6]. For most carcinomas, progression toward malignancy is accompanied by loss of epithelial differentiation and a shift towards a mesenchymal phenotype [7]. It is well-established that this occurs through epithelial-mesenchymal transition (EMT), the mechanism believed to help tumour cells in their ability to migrate, invade, and metastasize [8, 9]. EMT is an essential morphologic conversion that occurs during embryonic development for complex body patterning. EMT occurs when epithelial cells lose their epithelial cell characteristics, including dissolution of cell-cell junctions (tight junctions, adherens junctions, and desmosomes), lose apical-basolateral polarity, and acquire a mesenchymal phenotype, characterized by actin reorganization and stress fiber formation, migration and invasion [10, 11]. The loss of cell-cell adhesion is a prerequisite of EMT, a hallmark of which is the functional loss of E-cadherin [4]. Emerging evidence suggests that EMT plays a crucial role in the progression

of EOC by increasing cancer cell motility, chemo-resistance, and cancer stem cell characteristics [12].

In EOC, signals from the neoplastic microenvironment, including a variety of cytokines and growth factors, function to control EMT. Although several growth factors participate in EMT, transforming growth factor-beta (TGFβ) has been the most studied. The TGFβ superfamily consists of a large number of structurally related polypeptide growth factors, including TGFβ, bone morphogenetic protein (BMP), inhibin/activin and Mullerian inhibiting substance (MIS) families; each is capable of regulating a broad spectrum of cellular processes, including cell proliferation, lineage determination, differentiation, motility, adhesion, embryogenesis, fibrosis, immunosuppression and apoptosis [13]. TGFβ signalling has been implicated in the pathogenesis of many different cancers, including EOC [9, 14]. Indeed, TGFβ is thought to play a role in the pathobiological progression of EOC because this cytokine is overexpressed in cancer tissue, plasma, and peritoneal fluid of ovarian cancer patients [15].

We have discovered that patient ascites-derived EOC cells naturally undergo a robust EMT response by simply aggregating into spheroids, and this was reversed upon spheroid re-attachment to a substratum. Given that TGFβ is a key regulator of EMT in carcinomas [16], we investigated its role in spheroid-induced EMT and found that treatment with the TβRI inhibitor, SB-431542, potently blocked endogenous EMT in spheroids. Additionally, SB-431542 treatment upon spheroid re-attachment further enhanced the epithelial phenotype of dispersing cells while decreasing cell motility and migration. In fact, spheroid formation was compromised by exposure to SB-431542, and this rendered cells susceptible to carboplatin-induced cell death.

2.2 Material and Methods

2.2.1 Cell culture

Ascites fluid obtained from ovarian cancer patients at the time of debulking surgery or paracentesis was used to generate primary cell cultures as described previously [17]. Briefly, bulk ascites containing cells was mixed 1:1 with growth medium [MCDB105

(Sigma, St. Louis, MO) / M199 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Wisent, St. Bruno, Quebec, Canada) and 50 µg/ml penicillin-streptomycin]. Cells were grown in a 37°C humidified atmosphere of 95% air and 5% CO_2. To account for inter-patient variability and the finite lifespan of primary ascites-derived cells, many independent samples collected from patients with high-grade EOC were used throughout the study (Supplementary Table S1). Experiments were performed using cells between passages 3 and 5 and performed with at least four independent patient samples and at least three experimental replicates. Samples have been confirmed independently to have greater than 90% EpCAM-positive cells beyond passage 2. All patient-derived cells were used in accordance with institutional human research ethics board approval (UWO HSREB 12668E).

Adherent cells were maintained on tissue culture-treated polystyrene (Sarstedt, Newton, NC). Non-adherent cells (spheroids) were maintained for 72 h on Ultra-low Attachment (ULA) cultureware (Corning, Corning, NY) which is coated with a hydrophilic, neutrally charged hydrogel to prevent cell attachment. For spheroid re-attachment assays, spheroids were removed from ULA cultureware and re-introduced to tissue culture-treated polystyrene for up to 72 h to allow for cell dispersion.

2.2.2 **Quantitative RT-PCR**

Total RNA was isolated from primary adherent and spheroid EOC cells using Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA). RNA was isolated from reattached spheroids by first trypsinizing cells and spheroids from the tissue culture plastic. Purified RNA was quantified using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Reverse transcription was performed using total RNA isolated from independent patient samples (adherent and/or spheroid, treated and untreated) and Superscript II reverse transcriptase (Invitrogen) as per manufacturer's instructions. PCR reactions were carried out using Brilliant SYBR Green QPCR Master Mix (Agilent Technologies/Stratagene) and a Stratagene Mx3000P machine with data exported to Microsoft Excel for analysis. Human-specific primers for *CDH1* (E-cadherin), *SNAI1* (Snail), *TWIST1*, *TWIST2*, *ZEB2*, and *VIM* (Vimentin) were used (Sigma). *GAPDH* served

as an internal control for RNA input and quantification was performed using the ΔΔCt method [18].

2.2.3 Western blot analysis

Total cellular protein was isolated from primary adherent and spheroid EOC cells. Cells were washed once in cold phosphate-buffered saline (PBS), dissolved in lysis buffer [50 mM HEPES pH7.4, 150 mM NaCl, 10% glycerol, 1.5 mM MgCl2, 1mM EGTA, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 10 mM NaF, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 1x protease inhibitor cocktail (Roche, Laval, Quebec, Canada)], clarified by centrifugation (20 min at 15,000g), and quantified by Bradford analysis (Bio-Rad Laboratories, Mississauga, Ontario, Canada). Nuclear fractions were isolated from primary adherent and spheroid EOC cells by washing cells in PBS, resuspending the cell pellet in a hypotonic lysis buffer [20 mM HEPES (pH 7.4), 1 mM EGTA, 1 mM EDTA, 1 mM DTT] for 15 min on ice. Lysates were centrifuged, supernatant removed, and nuclear pellet washed twice with wash buffer [10 nM HEPES (pH 7.4), 10 mM KCl, 0.1 mM EGTA, 0.1 mM EDTA]. The washed nuclear pellet was dissolved in lysis buffer and clarified by centrifugation as described above for total cellular protein isolation. Forty to fifty micrograms of protein extract per lane were separated by SDS-PAGE in the presence of 1% β-mercaptoethanol using 8% or 10% gels. Proteins were then transferred to a polyvinylidene difluoride membrane (PVDF; Roche, Laval, Quebec, Canada), blocked with 5% BSA in Tris-buffered saline with Tween-20 [TBST; 10 mM Tris.HCl, pH 8.0, 150 mM NaCl, 0.1% Tween-20]. Membranes were washed in TBST and incubated (overnight, 4 ºC) with appropriate antibodies (1:1000 in 5% BSA/TBST). Immunoreactive bands were visualized by incubating (1 h, room temperature) with peroxidase-conjugated anti-rabbit or anti-mouse IgG (1:10,000 in 1% skim milk/TBST; GE Healthcare) followed by exposure to enhanced chemiluminescence reagent (Luminata^{TM} Forte, Millipore).

2.2.4 Antibodies and other reagents

Antibodies against E-cadherin (#3195), Snail (#3879), β-Catenin (#9582P), Claudin-1 (#4933), Smad2 (#3122) and phospho-Smad2 (#3108S) were purchased from

Cell Signalling Technologies (Danvers, MA). Anti-human Lamin A+C antibody (MAB3211) was purchased from Millipore (Temecula, CA). Anti-actin antibody (A2066) and anti-α-tubulin (T5168) were purchased from Sigma (Mississauga, ON). Recombinant human TGFβ1 was purchased from Millipore (Temecula, CA) and prepared in distilled water, and used at a concentration of 10 ng/mL [9]. The TGFβ receptor I (TβRI) inhibitor, SB-431542, was purchased from Sigma and prepared in DMSO according to manufacturer's instructions, and used at a concentration of 5 μM [19].

2.2.5 **TGFβ signalling modulation**

To test TGFβ responsivity of primary EOC cells, adherent cells were treated with recombinant human TGFβ1 at 2, 24, and 72 h in reduced serum media (0.5% FBS). Cells were harvested for analysis at each time point and images were captured using a Leica DMI 4000B inverted microscope. Spheroids were treated with SB-431542 or dimethyl sulphoxide (DMSO) vehicle control at the time of initial seeding to ULA culture ware. Seventy-two hours later, spheroids were imaged and harvested for analysis. For spheroid re-attachment assays, native (untreated) spheroids were removed from ULA cultureware and re-introduced to tissue culture-treated polystyrene for 72 h to allow for cell dispersion. SB-431542 treatment or DMSO control was given at the time of re-attachment to tissue culture-treated plates and harvested 72 h later for analysis.

2.2.6 **Spheroid re-attachment assay**

Macroscopically-visible spheroids (formed over 3 d) were transferred individually into single wells of 48-well tissue culture-treated polystyrene plates (12 replicates per treatment per EOC sample) and treated with DMSO or SB-431542 at the time of re-attachment. Phase contrast images of each well were captured using Leica DMI 4000B inverted microscope at 24 h and dispersion area was quantified using *ImageJ* (NIH, Bethesda, MD), as described previously (Peart et al., 2012) Area of dispersion for each re-attached spheroid was determined as total dispersion area minus spheroid (core) area at 24 h. At 72 h, re-attached spheroids were fixed and stained using Hema-3 Stain kit (Fisher, Kalamazoo, MI).

2.2.7 Transwell migration assay

Primary EOC cells were seeded at 5 x 10^4 cells per well in 24-well ULA plates and treated with either DMSO or SB-431542 at the time of seeding. EOC spheroids were washed in PBS and transferred individually to 100 µL of reduced serum media (0.5% FBS) and added to the top chamber of a Transwell (6.5 mm in diameter; 8 µm pore size) (Costar®), with 600 µL of medium containing 10% FBS added to the bottom chamber of well. After a 12 h incubation, Transwell membranes were fixed and stained with Hema-3 Stain kit and non-migrated cells on the upper side of the membrane were removed completely using a cotton swab. Migrated cells on the underside of the membrane were imaged with a Leica DMI 4000B inverted microscope. Images in five different fields per membrane were captured and cells were counted with *ImageJ* software. The mean number of migrated cells was determined for each Transwell.

2.2.8 Immunofluorescence

Analysis of F-actin and β-catenin protein by fluorescent staining was performed on re-attached EOC spheroids seeded to glass coverslips and treated with SB-431542 or DMSO vehicle control at the time of spheroid re-attachment. After 72h, cells were fixed in a buffered 10% formalin solution and spheroid cores were removed leaving only the cells that have dispersed out of the spheroids attached onto the coverslips for analysis. Cells were washed with PBS and permeabilized with 0.1% Triton X-100 in PBS. Overnight incubation with anti-β-catenin antibody (1:500) was followed by one hour incubation with anti-rabbit fluorescein isothiocyanate-conjugated secondary antibody (1:500; Vector Laboratories) and subsequently a one hour stain with Alexa Fluor® 568-conjugated phalloidin (A12380, Invitrogen). Coverslips were mounted in VectaShield mounting medium (Vector Laboratories) containing 4′,6-diamidino-2-phenylindole (1:1000; Sigma, St. Louis, MO). Fluorescence images of mounted coverslips were captured using a Leica DMI 4000B inverted microscope.

2.2.9 Cell viability

Cells were seeded to either 24-well tissue culture plastic at a density of 1 x 10^4 per well to form adherent cultures, or ULA plates at a density of 5 x 10^4 per well to form spheroids. SB-431542 treatment was initiated at the time of seeding for cells in suspension, while cells under adherent conditions were given 12 h to adhere before commencing treatment. At 72 h post-treatment, spheroids were collected, pelleted and left in media (100 µL), at which point CellTiter-Glo® reagent (Promega, Madison, WI) was added (1:1 v/v ratio). All samples were subjected to a freeze/thaw cycle prior to analysis. Approximately 100 µL of the mixture was added to a white-walled 96-well microplate and luminescence signal was detected using a Wallac 1420 Victor 2 spectrophotometer (Perkin-Elmer, Waltham, MA) and normalized to vehicle-treated cells. Under adherent conditions, cells were kept in original 24-well plates and cell viability was assessed using alamarBlue® (Invitrogen, Burlington, ON) as per manufacturer's instructions. Briefly, medium was replaced with alamarBlue® reagent diluted 1:20 in complete medium, incubated for 4 h at 37 °C in a humidified atmosphere of 95% air and 5% CO_2, and then fluorescence was quantified using 560/590 nm excitation/emission filter settings.

For measuring spheroid cell viability in the presence of carboplatin, spheroids were treated first with SB-431542 or DMSO for 72 h, followed by 100 µM carboplatin (LHSC Pharmacy, London, ON); CyQUANT® NF (Invitrogen, Burlington, ON) assays were performed 72 h later. Briefly, spheroids were collected and pelleted, and medium was removed. After a single freeze-thaw, spheroids were resuspended in 1X Hank's balanced salt solution (HBSS), and 50 µL aliquots were dispensed into microplate wells. Fifty microlitres of 2X dye binding solution was added to each microplate well, and fluorescence signal was detected with the Wallac plate reader using 485/530 nm excitation/emission filter settings.

2.2.10 Statistical analysis

Data were expressed as mean ± SEM. All statistical analyses were performed using a two-tailed Student's *t*-test or one-way analysis of variation (ANOVA) with Tukey's Multiple Comparison test. Significance levels were set at $p < 0.05$.

2.3 Results

2.3.1 EMT is induced during EOC spheroid formation

To examine EMT in EOC ascites-derived cells, we followed the expression profile of the epithelial marker E-cadherin and mesenchymal markers known to repress E-cadherin (Snail, Twist1, Twist2, Zeb2) during spheroid formation and reattachment [20, 21]. We used an *in vitro* 3D culture system whereby primary ascites-derived human EOC cells are maintained in suspension on ultra-low attachment plates for 3 days,followed by re-introduction to standard tissue culture-treated plates where they rapidly re-attach and disperse [22, 23]. Accordingly, we performed quantitative RT-PCR analysis on RNA isolated from several independent patient samples (n=6) that were grown in adherent, spheroid, and re-attached spheroid culture conditions. Compared to their matched adherent counterparts, primary EOC ascites cells were found to naturally undergo an EMT response during spheroid formation based on mRNA upregulation of *SNAI1*, *TWIST1*, *TWIST2*, *ZEB2* and a coordinate down-regulation of *CDH1* (Figure 2.1A). Of these mesenchymal markers, *SNAI1* was the most robustly and consistently upregulated EMT marker in spheroids (11.7 - 42.5 fold increase). Several of these trends were strongly reversed upon EOC spheroid re-attachment (after 3 d re-attachment), as the mRNA levels of *SNAI1*, *TWIST1*, *TWIST2*, and *ZEB2* return to levels comparable to those of matched adherent cells. Although *CDH1* transcript levels begin to recover, they did not fully attain expression levels of original adherent cells in this model system.

Western blotting was performed using protein lysates isolated from independent EOC ascites-derived human cells grown in the same culture conditions to verify the trends seen at the transcript level. In EOC spheroids, E-cadherin protein levels were down-regulated and this was associated with a robust up-regulation of Snail. These effects started to reverse upon EOC spheroid re-attachment, although E-cadherin protein expression was not restored in most patient samples by 3 d after re-attachment (Figure 2.1B). Thus, RNA and protein expression data demonstrate EOC ascites-derived cells undergo endogenous EMT during spheroid formation, and this phenomenon is triggered to reverse upon spheroid re-attachment.

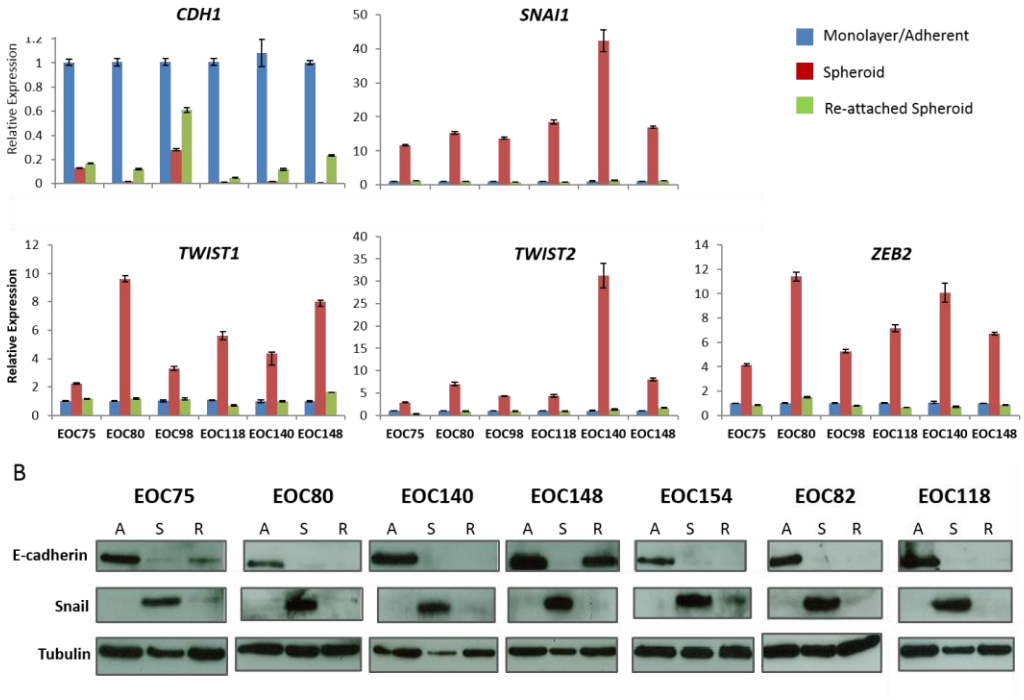


Figure 2.1: EMT is induced during EOC ascites cell spheroid formation and reversed upon re-attachment.
(A) Quantitative RT-PCR analysis of *CDH1*, *SNAI1*, *TWIST1*, *TWIST2*, and *ZEB2* mRNA in monolayer/adherent cells, spheroid cells, and 72 h re-attached spheroid cells using primary ascites-derived EOC patient samples (n=6). **(B)** Western blot analysis of E-cadherin and Snail protein in adherent [A], spheroid [S] and 72 h re-attached spheroid samples [R] samples in primary ascites-derived EOC patient samples (n=7). Tubulin was used as a loading control.

2.3.2 TGFβ signalling regulates EMT in primary ascites-derived EOC spheroids

To address the potential mechanism by which EOC cells undergo endogenous EMT upon spheroid formation, we chose to assess the role of the TGFβ signalling pathway in mediating the pathobiological characteristics of EOC ascites spheroids. Previous microarray data from our lab [22] revealed increased *TGFβ1* expression in EOC spheroids compared to adherent cells in five independent patient ascites samples (mean 4.36-fold, $p<0.001$).

First, we tested the responsivity of primary EOC cells in adherent culture to TGFβ1 treatment. In doing so, patient ascites samples (n=4) were treated with 10 ng/mL recombinant TGFβ1 ligand for 2, 24, and 72 h (Figure 2.2A) and quantitative RT-PCR analysis was performed. Treatment of adherent cells with TGFβ1 ligand resulted in a significant decrease of *CDH1* mRNA at 24 h, but returned to near basal levels by 72 h. However, we observed significant increases in mesenchymal marker transcript levels for *SNAI1*, *ZEB2*, *TWIST2*, and *VIM* at 24 and 72 h. Moreover, transition of cells from a typical cobblestone epithelial morphology to a distinctly fibroblast-like phenotype was observed after 72 h of TGFβ1 treatment (Figure 2.2B), indicating induction of EMT and responsiveness of EOC ascites-derived cells to TGFβ.

EMT was shown to be induced endogenously upon spheroid formation in primary ascites-derived EOC cells (Figure 2.1). Thus, we wanted to assess whether further treatment with exogenous TGFβ1 ligand would potentiate EMT phenotype induced in spheroids, and compare endogenous EMT induction in EOC spheroids to TGFβ1-induced EMT in adherent EOC cells. Quantitative RT-PCR analysis revealed no significant changes in *CDH1*, *SNAI1*, *VIM*, *TWIST2*, or *ZEB2* mRNA levels in TGFβ1-treated EOC ascites spheroids relative to untreated spheroids (data not shown). Western blot analysis confirmed that treatment of spheroids with TGFβ1 does not further increase Snail protein levels (Figure 2.2C). Furthermore, there were higher levels of Snail protein in untreated spheroids compared to TGFβ1-treated EOC ascites cells indicating that spheroid formation alone may act as a potent inducer of EMT. We also observed that TGFβ1 treatment of adherent

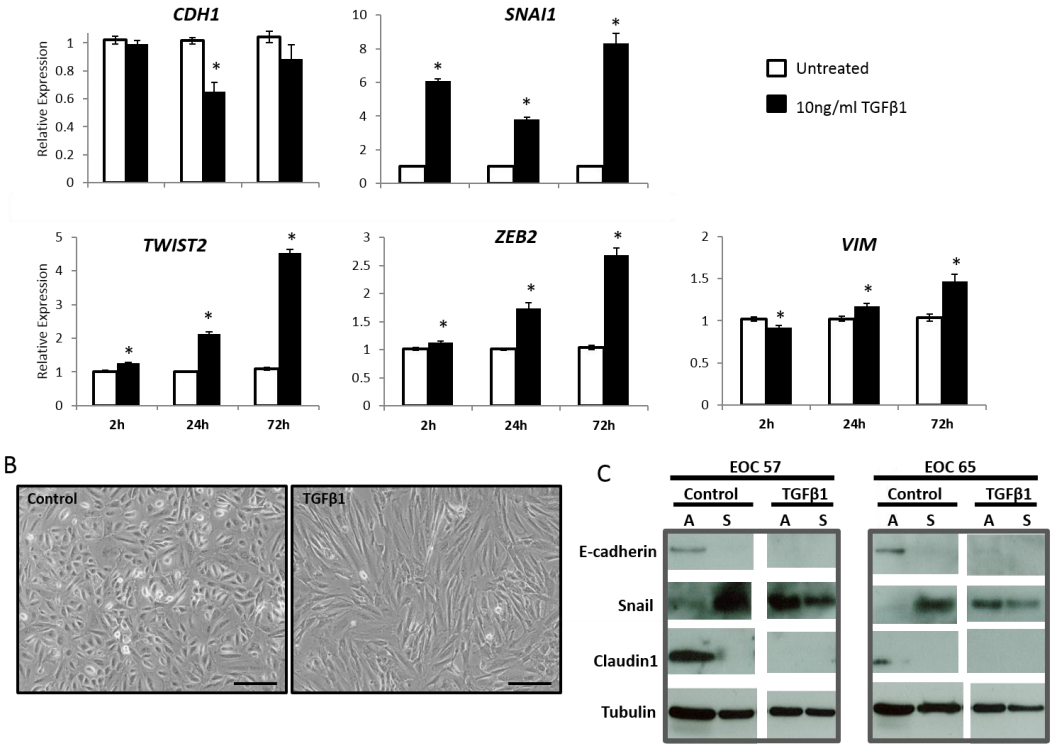


Figure 2.2: TGFβ induces EMT in EOC ascites-derived cells but spheroid formation alone is a more potent inducer of EMT.
(A) Quantitative RT-PCR analysis of *CDH1*, *SNAI1*, *TWIST2*, *ZEB2* and *VIM* mRNA in control and TGFβ1-treated adherent EOC ascites-derived cells (n=4) at 2 h, 24 h, and 72 h (*, $p<0.05$ as determined by Student's *t*-test). **(B)** TGFβ1 treatment of EOC ascites-derived cells induces transformation of epithelial cells towards a fibroblastic mesenchymal morphology. Representative image from EOC57 patient sample at 72 h post treatment (scale bar=200 µm). **(C)** Western blot analysis of E-cadherin, Claudin1, and Snail protein in adherent [A] and spheroid [S] samples treated with TGFβ1 or not in ascites-derived EOC patient samples (n=2). Tubulin was used as a loading control. Panels represent separate lanes from the same blot and same length of exposure.

cells results in sustained reduction in E-cadherin protein as compared with the slight recovery in *CDH1* mRNA level at 72 h.

Because we have observed increased TGFβ1 expression in EOC ascites-derived spheroids, we propose that endogenous TGFβ signalling activity regulates the natural induction of EMT observed in spheroids. In support of this, we have observed increased nuclear phosphorylated Smad2 and total Smad2 protein in spheroid cells as compared with adherent ascites cells (Figure 2.3). As such, we hypothesize that inhibiting TGFβ signalling in EOC ascites-derived cells in suspension will abrogate spheroid formation and induction of EMT. Inhibiting TGFβ signalling during spheroid formation using a TβRI small molecule inhibitor, SB-431542, efficiently blocked the EMT phenotype in spheroids as evidenced by a significant increase in *CDH1* mRNA expression and a significant decrease in *SNAI1*, *TWIST2*, and *ZEB2* mRNA expression (Figure 2.4A). Increased E-cadherin protein and decreased Snail protein expression were consistently seen in SB-431542-treated spheroids compared to DMSO controls, indicating EMT is dramatically reduced in spheroids as a result of inhibiting TGFβ signalling (Figure 2.4B). In fact, SB-431542 treatment resulted in spheroids that were much smaller in size and less cohesive than the compact control spheroids (Figure 2.4C), which resulted in decreased viability as compared with adherent cells (Figure 2.4D). We confirmed that SB-431542 treatment of adherent cells and spheroids resulted in blockade of TGFβ1-induced signalling by phospho-Smad2 western blotting (Figure 2.5). In addition, SB-431542 treatment of adherent EOC ascites cells increases E-cadherin protein expression and promotes an exaggerated epithelial phenotype (data not shown).

2.3.3 TGFβ signalling inhibition during EOC spheroid re-attachment enhances the epithelial phenotype of dispersing cells and reduces cell motility

A common feature of EMT in cancer is the enhanced capacity for malignant cells to migrate; and the capacity of EOC ascites cell spheroids to re-attach, grow, and disperse defines their ability to form secondary metastases [5]. Since inhibition of TGFβ signalling consistently blocks EMT and reduces cell-cell cohesion within spheroids, we sought to

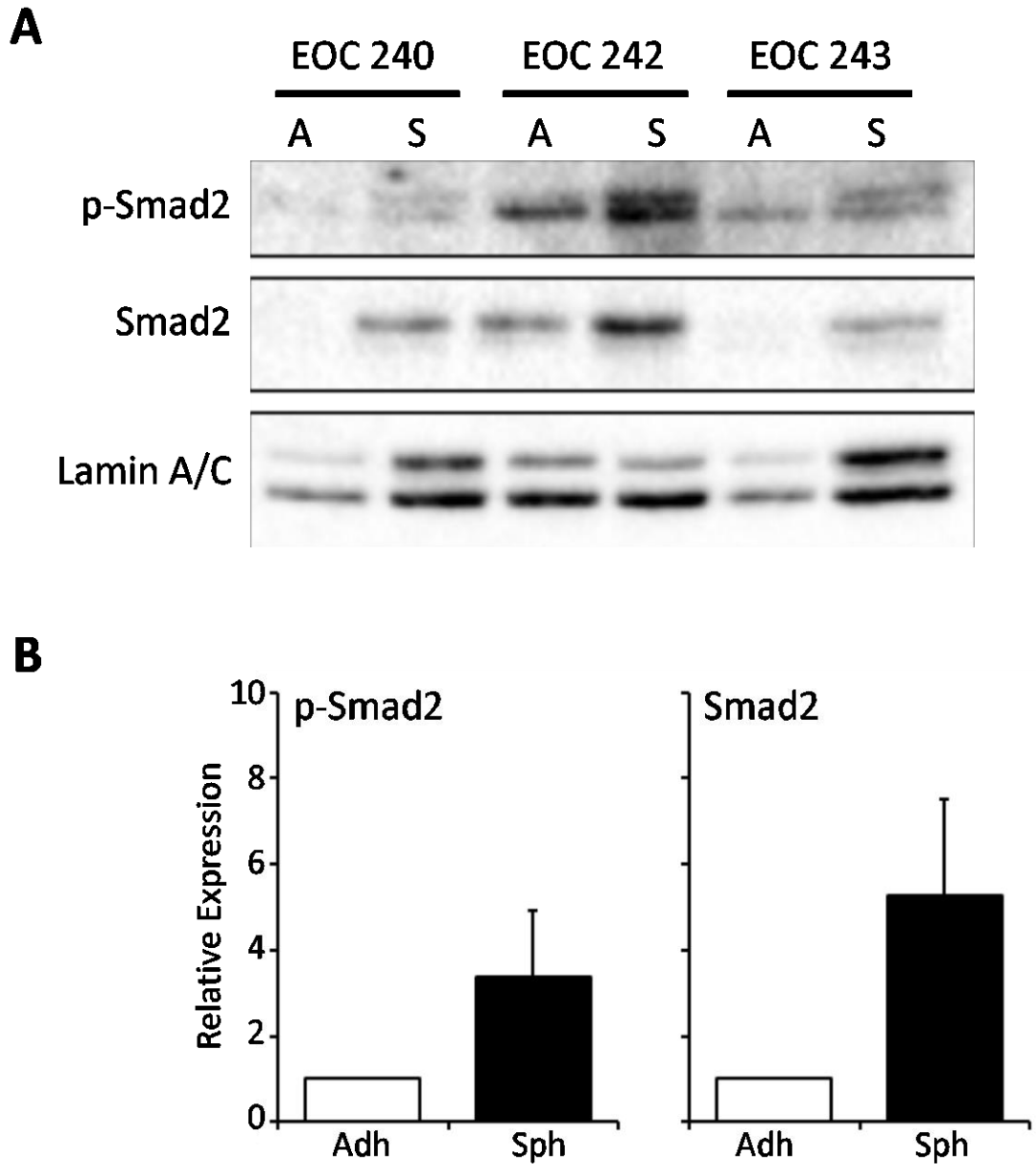


Figure 2.3: Increased nuclear Smad2 indicating activated TGFβ signalling in spheroids.
(A) Immunoblot analysis of phospho-Smad2 and total Smad2 on nuclear protein extracts isolated from EOC patient-derived ascites specimens cultured as adherent cells [A] or spheroids [S] (n=3). Lamin A/C was used as loading control. (B) Quantification of mean phospho-Smad2 and total Smad2 expression from the above immunoblots as normalized to Lamin A/C.

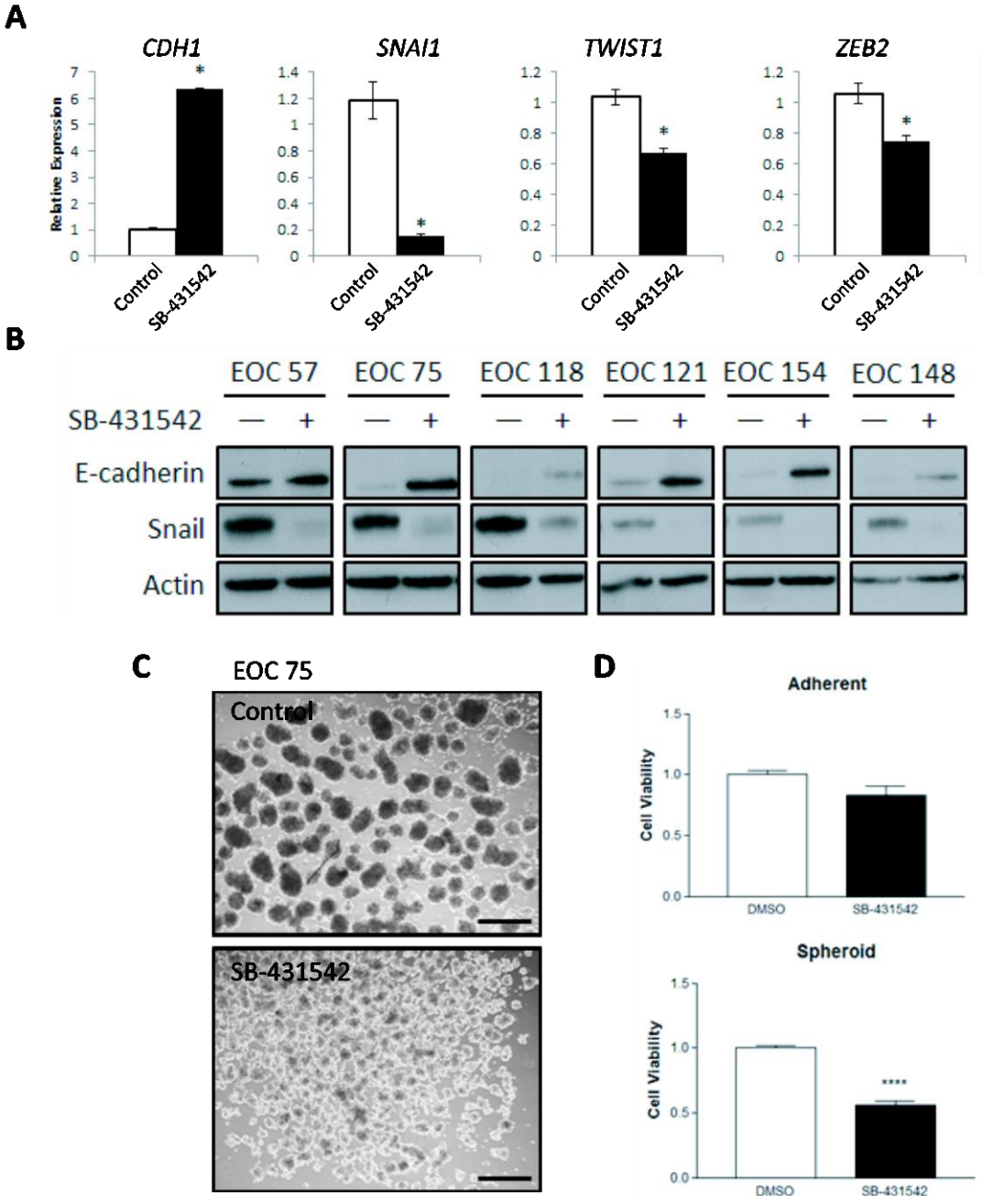

A
CDH1
SNAI1
TWIST1
ZEB2
Relative Expression
Control
SB-431542
B
EOC 57
EOC 75
EOC 118
EOC 121
EOC 154
EOC 148
SB-431542
E-cadherin
Snail
Actin
C
EOC 75
Control
SB-431542
D
Adherent
Cell Viability
DMSO
SB-431542
Spheroid
****

Figure 2.4: Inhibition of TGFβ signalling in spheroids blocks EMT and compromises EOC spheroid morphology.
(A) Quantitative RT-PCR analysis of *CDH1*, *SNAI1*, *TWIST2*, and *ZEB2* mRNA in control and SB-431542-treated spheroids (n=7; *, $p<0.05$ as determined by Student's *t*-test). (B) Western blot analysis of E-cadherin and Snail protein in spheroids formed from EOC ascites-derived patient samples (n=6). Actin was used as a loading control. (C) SB-431542 treatment of EOC ascites-derived cells at the time of seeding to ULA forms smaller and less cohesive spheroids compared to DMSO controls. Representative image from EOC75 patient sample at 72 h post treatment (scale bar=500 µm). (D) Cell viability was determined using CellTiter-Glo® assay following 3 d of SB-431542 treatment, or DMSO control across EOC patient ascites samples (n=8) cultured as adherent cells and spheroids. Data is represented as mean ± SEM and Student's *t*-test for statistical significance (***, $p<0.001$).

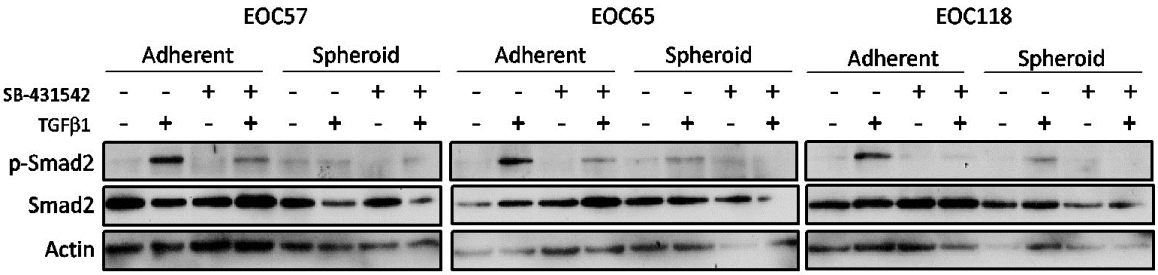


Figure 2.5: SB-431542 treatment of EOC adherent cells and spheroids blocks TGFβ1-induced signalling

Immunoblot analysis of phospho-Smad2 and total Smad2 on whole-cell protein extracts isolated from EOC patient-derived ascites specimens (n=3). EOC samples were serum-starved overnight and pre-treated with SB-431542 for 30 min, and subsequently treated with 10 ng/mL TGFβ1 for 30 min. Actin was used as loading control.

determine whether TGFβ signalling inhibition affects the ability of spheroids to re-attach and disperse. Thus, our first assessment was to quantify cell migration from SB-431542-treated spheroids across a Transwell membrane. We observed that blockade of TGFβ signalling significantly reduced the ability of cells (n=9) to migrate out of spheroids across a Transwell membrane (Figure 2.6). Since we observed that SB-431542 treatment of spheroids reduces cell viability (Figure 2.3D), we could not attribute these Transwell assay results to migration only. Therefore, we also used spheroid re-attachment assays to quantify cell motility, where only viable spheroid cells will re-attach and disperse. Spheroids formed over 3 d from patient ascites samples (n=7) were plated individually for re-attachment using standard tissue culture- treated plates. Treatment with SB-431542, or DMSO control, was started at the time of seeding for re-attachment. Remarkably, we observed less-cohesive spheroid cores within 24 h of SB-431542 treatment (Figure 2.7A). We also observed a significant decrease in cell dispersion area in SB-431542-treated spheroids compared with DMSO controls (Figure 2.7B). Since we demonstrated that SB-431542 treatment did not affect ascites cell proliferation over 72 h (Figure 2.3D), this indicates that decreased dispersion from reattached spheroids is due to reduced cell motility via TGFβ signalling inhibition.

Since inhibiting TGFβ signalling in re-attached spheroids decreased cell motility of dispersing cells, we postulated that this was due to an enhanced epithelial phenotype of dispersing cells. Indeed, EOC ascites-derived cell spheroids treated with SB-431542 during re-attachment yielded dispersing cells exhibiting an enhanced epithelial morphology compared with DMSO controls (Figure 2.7C). To assess the epithelial morphology of cells dispersing from re-attached spheroids further, we performed immunofluorescence staining for several cytological markers. Since it was difficult to obtain membrane-specific immunofluorescence staining of E-cadherin, we chose to assess β-catenin. β-catenin is an integral component of the protein complex in adherens junctions and binds to the E-cadherin receptor intracellularly anchoring it to the actin cytoskeleton [24, 25]. Furthermore, β-catenin protein expression was up-regulated in re-attached spheroids treated with SB-431542 by Western blot analysis (data not shown). Immunofluorescence

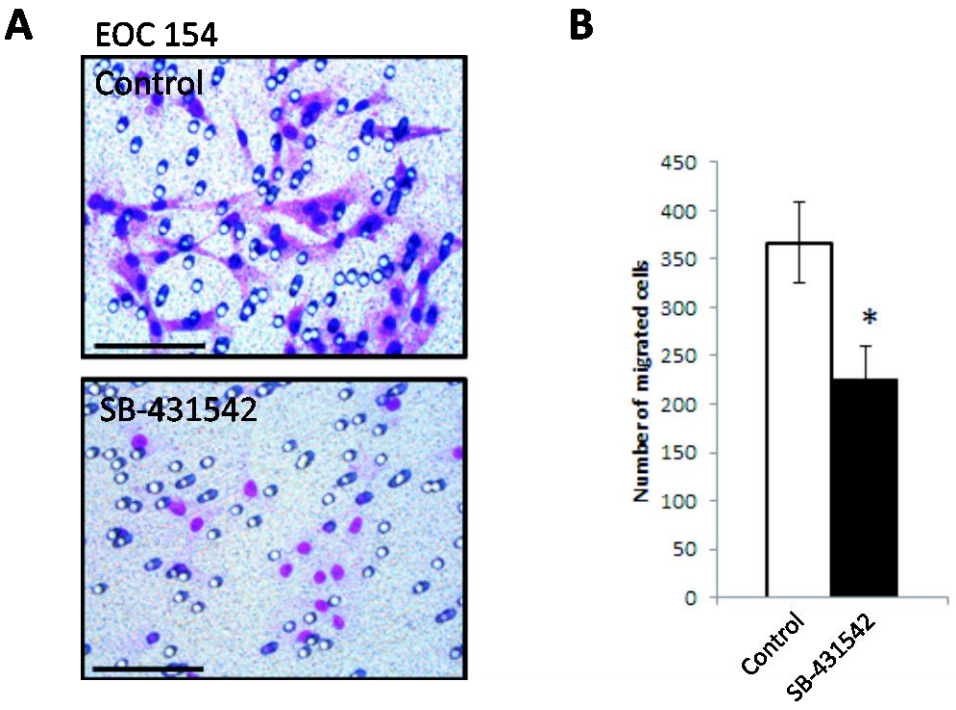


Figure 2.6: Inhibition of TGFβ signalling in spheroids decreases migration of cells across a Transwell.
(A) SB-431542 treatment of EOC ascites-derived cells at the time of seeding to ULA and transfer of day 3 spheroids to Transwell inserts decreases cell migration as compared with DMSO-treated controls. Representative EOC154 patient sample showing fewer migrated cells in SB-431542 treated spheroids compared to DMSO controls (scale bar=100 μm). (B) Transwell cell migration of treated spheroids from patient-derived samples (n=9) quantified using *ImageJ* software and averaged amongst 5 different fields per image (*, $p<0.05$ as determined by Student's *t*-test).

images showed enhanced plasma membrane localization of β-catenin in cells dispersing out of SB-431542 treated re-attached spheroids compared to DMSO controls (Figure 2.7D). Moreover, phalloidin staining for F-actin showed reduced stress fiber formation in SB-431542 treated re-attached spheroids (Figure 2.7E), also indicative of an enhanced epithelial phenotype of dispersing cells. To further validate this at the molecular level, we performed quantitative RT-PCR analysis of RNA isolated from re-attached spheroids from several independent EOC patient ascites samples. Re-attached spheroids treated with SB-431542 showed a significant up-regulation of *CDH1* mRNA and this was associated with a down-regulation of *SNAI1* (Figure 2.8A). This was further validated by western blot analysis which showed enhanced E-cadherin protein expression associated with reduced Snail protein expression (in 5 out of 6 re-attached EOCs) in SB-431542 treated re-attached spheroids compared to DMSO controls (Figure 2.8B).

2.3.4 SB-431542 treatment of spheroids potentiates carboplatin-induced cell death

Platinum-based chemotherapy is the standard for first-line treatment of metastatic epithelial ovarian cancer, yet the majority of patients will eventually recur with platinum-resistant disease [26]. Ovarian cancer spheroids are largely resistant to platinum treatment, likely due to their reduced proliferative state. To assess whether blocking TGFβ signalling can affect ovarian cancer spheroid sensitivity to platinum agents, we treated EOC spheroids with SB-431542 or vehicle control first, followed by treatment with carboplatin. We observed a dramatic reduction in viability in EOC ascites-derived cell spheroids that received a combined treatment of SB-431542 at day 0 followed by 72h carboplatin treatment at day 3, when compared to either SB-431542 treatment alone or a combined treatment of vehicle plus carboplatin (Figure 2.9A,B). These final results point to a critical role for TGFβ signalling in maintaining cell viability and achieving chemo-resistance in ascites-derived cell spheroid.

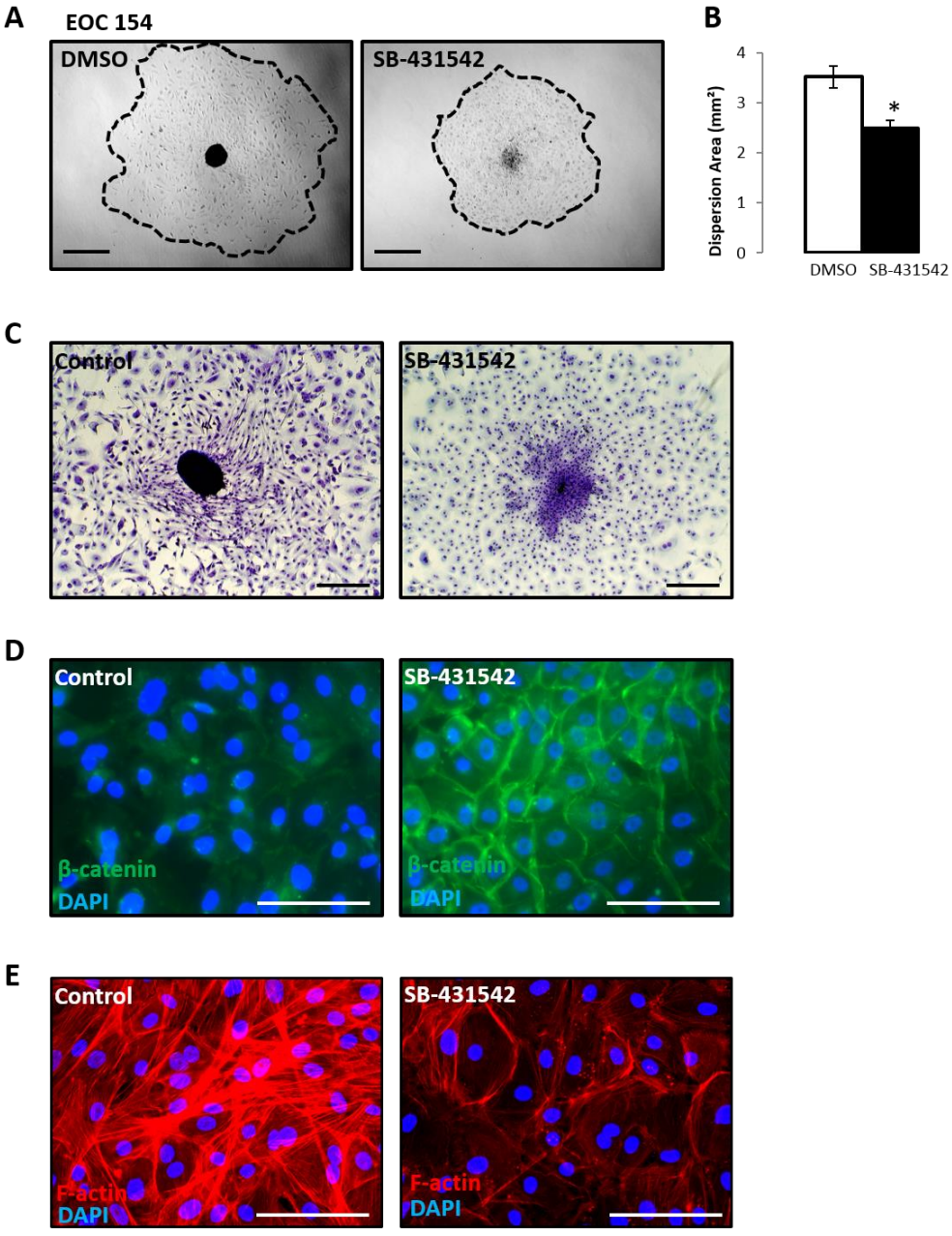

A
EOC 154
DMSO
SB-431542
B
Dispersion Area (mm²)
4
3
2
1
0
*
DMSO
SB-431542
C
Control
SB-431542
D
Control
β-catenin
DAPI
SB-431542
β-catenin
DAPI
E
Control
F-actin
DAPI
SB-431542
F-actin
DAPI

Figure 2.7: Inhibition of TGFβ signalling in re-attached spheroids decreases motility of dispersing cells and enhances their epithelial phenotype.
(A) SB-431542 treatment of EOC spheroids started at the time of re-attachment to standard tissue culture plates disrupts the spheroid core and decreases cell dispersion area. Representative image from EOC154 patient sample at 24 h post treatment (scale bar=500 μm). (B) Dispersion area was quantified using *ImageJ* software and averaged amongst 12 replicates per treatment condition (SB-431542 or DMSO control) for each EOC patient sample (n=7). Dispersion area was calculated 24 h after spheroids had been re-plated to standard tissue culture plastic (*, $p<0.05$ as determined by Student's *t*-test). (C) SB-431542 treatment of spheroids started at the time of re-attachment to standard tissue culture plates disrupts EOC spheroid core and changes morphology of dispersing cells to a more cuboidal epithelial phenotype. Representative image from EOC154 patient sample that was Hema-3-stained at 72 h post treatment (scale bar=500 μm). (D,E) Immunofluorescence images of cells dispersing out of SB-431542 treated re-attached spheroids shows enhanced β-catenin staining and reduced stress fiber formation (by rhodamine-phalloidin staining) compared to DMSO controls (scale bar=100 μm).

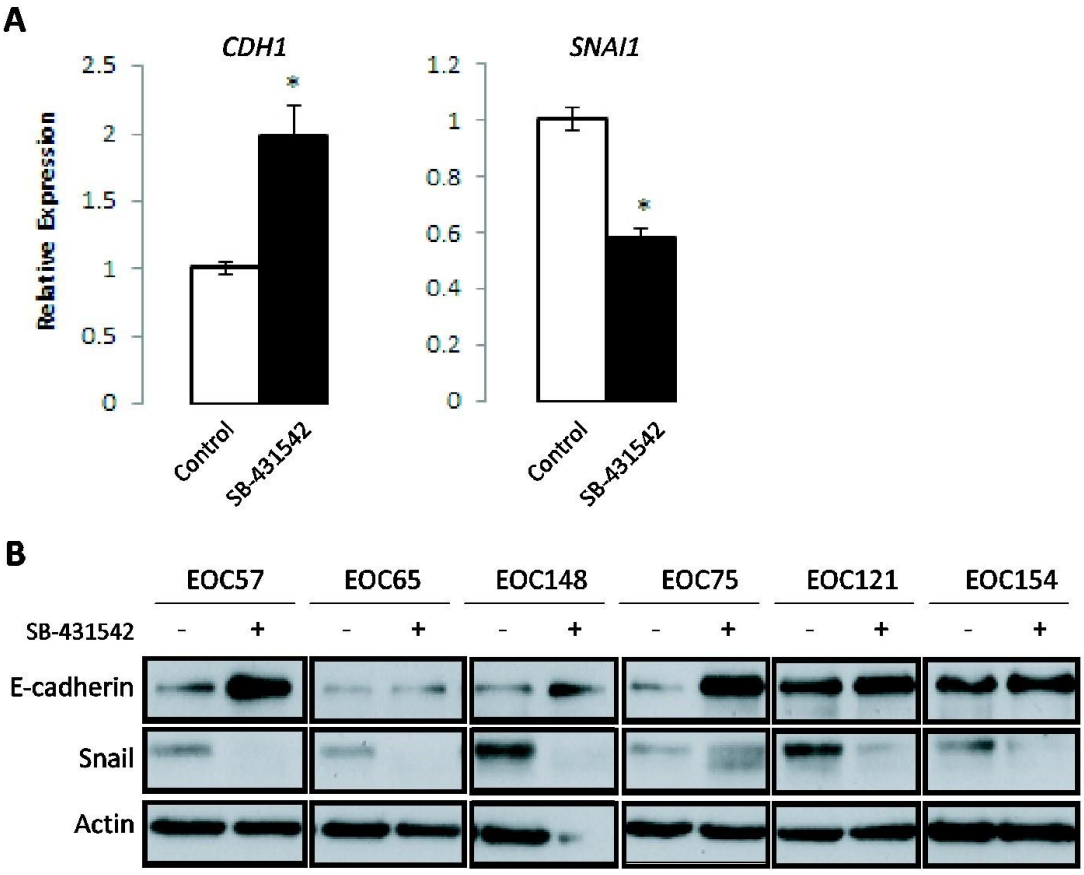
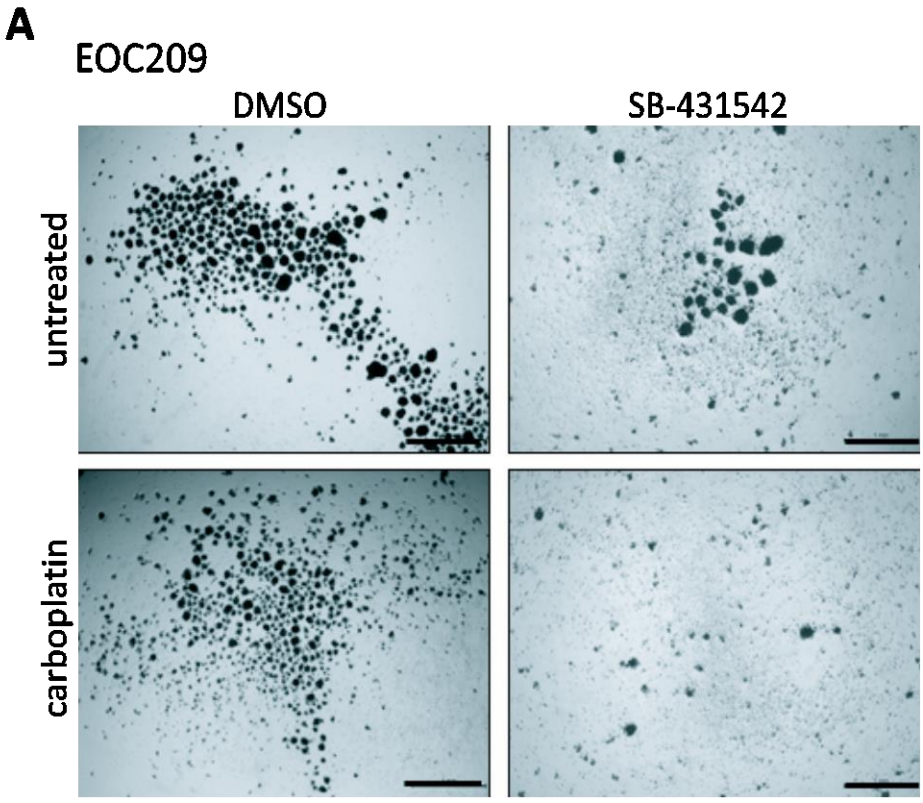
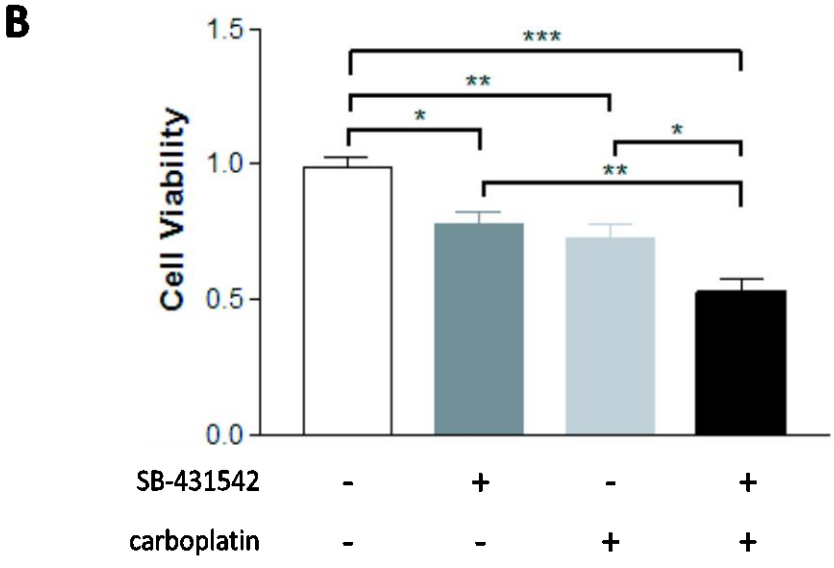


Figure 2.8: E-cadherin and Snail expression in SB-431542 treated re-attached spheroids

(A) Quantitative RT-PCR analysis of *CDH1* (E-cadherin) and *SNAI1* (Snail) in SB-431542-treated relative to DMSO-treated 24 h re-attached EOC spheroids (n=7; *, $p<0.05$ as determined by Student's *t*-test). (B) Western blot analysis of E-cadherin and Snail protein in 24 h re-attached spheroids in EOC ascites-derived samples (n=7). Actin was used as a loading control.


A
EOC209
DMSO
SB-431542
untreated
carboplatin


B
Cell Viability
1.5
1.0
0.5
0.0
***
**
*
*
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SB-431542 - + - +
carboplatin - - + +

Figure 2.9: TGFβ signalling in EOC ascites cells protects spheroids from platinum-induced cell death

(A) EOC ascites-derived cells (n=4) were treated with either DMSO or SB-431542 at the time of seeding to 24-well ULA cluster plate to form spheroids. Three days later, spheroids were treated with carboplatin (100 μM) for 72 h. Representative EOC209 patient sample showing reduced spheroid formation potential (fewer spheroids, more single cells) with combined carboplatin and SB-431542 treatment (scale bar=1 mm). (B) Cell viability was determined using CyQUANT® NF assay. Data is represented as mean ± SEM and one-way ANOVA with Tukey's Multiple Comparison test (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

2.4 Discussion

Spheroids have been postulated to act as important reservoirs of malignant EOC cells during spread throughout the peritoneal cavity in patients with late-stage disease [5, 27] We had previously observed that epithelial ovarian cancer cells induce an endogenous EMT response upon spheroid formation [22]. Herein, we have confirmed this phenomenon among a larger array of ascites-derived specimens and implicated the role of TGFβ signalling in its regulation. We also observed the plasticity of this process, since the cells undergo MET to revert back to an epithelial phenotype upon spheroid reattachment to a substratum. Importantly, we identified that intact TGFβ signalling is required to elicit this EMT response in spheroids since the potent small molecule type I receptor inhibitor SB-431542 blocks spheroid-induced EMT. SB-431542 treatment forces a more potent epithelial phenotype of dispersing cells from reattached spheroids, thereby reducing the spread of motile cells emanating from reattached spheroids. This also indicates that perhaps residual maintenance of TGFβ signalling acts to promote invasion and motility upon spheroid reattachment. Lastly, SB-431542 disrupts efficient EOC ascites cell spheroid formation and renders suspended cells more susceptible to carboplatin-mediated cell death. EMT has been observed in tumour cell spheroids and implicated in EOC metastasis, and we provide some essential insight into its regulation, as well as the therapeutic potential of targeting this important pathobiological process in late-stage EOC metastasis.

Epithelial to mesenchymal transition is a widely accepted phenomenon that occurs in the malignant progression of most if not all human carcinomas [28]. Dynamics of EMT during EOC metastasis, particularly with its reversal, or MET, upon secondary tumour formation, has been discussed in the literature [9, 11]. For example, E-cadherin down-regulation in ovarian cancer cells facilitates intraperitoneal spread [29], yet E-cadherin expression in metastases can be similar to the primary tumour [30]. We observed, however, that the level of E-cadherin expression upon spheroid re-attachment did not increase to initial levels completely, which may explain why secondary tumours oftentimes harbor low yet detectable E-cadherin in patients [31-33].

Differential E-cadherin expression supports the idea that switching between EMT and MET occurs during ovarian cancer metastasis and is important in disease progression. Our results provide further insight into the mechanism controlling this phenomenon of epithelial-mesenchymal plasticity within spheroids, which act as carriers of transiting metastatic cells in the peritoneal cavity. We demonstrate that the expression of classical EMT markers is rapidly induced during spheroid formation, yet the simple re-attachment to a substratum is sufficient to stimulate an MET cell phenotype. Our results imply that transcriptional repressors have immediate functions to induce EMT in spheroid formation and their reduction triggers the start of MET at reattachment since expression changes are rapid and sustained. It is peculiar that an EMT response would be induced in spheroids since these cells would not necessarily require a mesenchymal phenotype in these fairly static structures. However, one could envision that an EMT phenotype of spheroids primes the resident cells for invasive capabilities upon their subsequent reattachment [34]. On that note, E-cadherin is less immediately responsive particularly upon reattachment, perhaps indicating a delay to complete MET thereby facilitating spheroid cell motility and invasion. Induction of an EMT phenotype has been observed in other spheroid systems, usually in concert with stem-like cells [35, 36]; however, these reports have not described whether or not the EMT phenotype is reversible. It may be that this plasticity is a particularly unique characteristic of metastatic ovarian cancer, not unlike its almost exclusive mode of intraperitoneal spread which is unique when compared among other carcinomas.

The induced expression of EMT markers in spheroids implies that their gene products may act to facilitate disease progression. Snail expression in ovarian cancer portends poor patient prognosis [37] and platinum resistance [38]. Ectopic Snail expression in ovarian cancer cell lines enhances oncogenic transformation properties and promotes tumour growth in mouse xenografts, yet similar manipulation of Slug expression failed to elicit the same effect [39]. Snail and Slug may have different functions in controlling EMT in ovarian cancer cells [40, 41], which supports our findings that *SNAI2*/Slug expression is not consistently altered during spheroid formation and reattachment (data not shown). This exemplifies the utility of using our experimentally-tractable *in vitro* system using patient-derived cells not only to look at the dynamics of gene expression, but use it to dissect out the impact of these markers on spheroid cell biology. To this end, understanding Snail's

function and its requirement on the EMT phenotype in spheroids, anoikis-resistance, and cell invasion upon spheroid reattachment will be important. In addition, it will be important to determine whether TGFβ signalling directly regulates the genes controlling EMT, e.g. *SNAI1, TWIST1/2*, in EOC spheroid cells, as well as other pathobiological processes that this metastasis-associated pathway may regulate during spheroid formation and transcoelomic metastasis.

The TGFβ signalling pathway has been investigated for over 30 years in many human malignancies, including ovarian cancer [42]. Biologically-relevant concentrations of TGFβ ligands have been detected in malignant ascites from patients [43, 44]. Primary epithelial ovarian cancer cells possess an intact TGFβ signalling pathway, which can induce cytostasis upon its activation as long as *CDKN2B* encoding p15 is intact [44]. We showed previously that TGFβ signalling was present in spheroids [22]. This is consistent with TGFβ treatment regulating tissue transglutaminase 2 in ovarian cancer cells to promote EMT and spheroid formation [45]. In the present report, we have now uncovered a new function for active maintenance of endogenous TGFβ signalling in promoting the EMT phenotype of spheroids and their potential to reattach and spread. As such, TGFβ signalling may be essential to promote late-stage ovarian cancer progression, thus serving as a potential therapeutic target to reduce intraperitoneal spread and disease burden upon recurrence.

Several attempts have been made in the past to test whether TGFβ signalling could be targeted with inhibitors in cancer treatment [46]. Perhaps the majority of these studies have looked at malignancies where metastasis had already occurred and thus the inhibitors may be less efficacious at this step of progression. Ovarian cancer represents an entirely different case, since after primary debulking and chemotherapy the majority of disease is reduced. The eventual recurrence of disease and the continual re-seeding of the peritoneal cavity with malignant cells imply that cyclic TGFβ signalling activity may be required to drive this process. Our results show that the underlying pathobiology of spheroid cells can be dramatically altered by treatment with the potent TGFβ type I receptor inhibitor SB-431542. Most importantly, we also demonstrate that TGFβ signalling blockade reduces EOC spheroid cell viability in concert with carboplatin treatment. It would be intriguing to

test the numerous other TGFβ signalling inhibitors available [47], and apply them in an *in vivo* intraperitoneal ovarian cancer metastasis model to offer further supportive evidence that the therapeutic potential of targeting TGFβ signalling can be re-evaluated in ovarian cancer.

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Table S2.1: Summary of ovarian cancer patient clinical data.

Sample	Age	Histological Subtype	Grade	Stage
EOC57	46	Serous	High	IIIC
EOC65	67	Serous	High	IIIC
EOC75	77	Serous	High	IIIC
EOC80	49	Serous	High	III
EOC82	44	Serous	High	IIIC
EOC98	31	Serous	High	IIIC
EOC117†	60	Clear cell	High	IIIC
EOC118	57	Serous	High	IIIC
EOC121	78	Serous	High	IIIC
EOC140	76	Serous	High	IIIC
EOC148	67	Serous	High	IC
EOC154	66	Serous	High	IIIC
EOC202	80	Serous	n.a.	IIC
EOC207*	65	Serous	High	n.a.
EOC209*	52	Serous	High	n.a.
EOC238	70	Serous	High	n.a.
EOC240	47	Serous	High	IIIC
EOC241†	60	Serous	Low	n.a.
EOC242	75	Serous	High	IIIC
EOC243	56	Serous	High	IIIC
EOC253*	63	Serous	High	n.a.
EOC261	61	Serous	High	IIIC
EOC262	73	Serous	High	IIIC
EOC281*	60	Serous	High	IIIC

†: One sample was from a patient with low-grade serous histology (EOC241) and one sample from a patient with clear cell histology (EOC117); the results using these two samples did not differ from the high-grade serous histologies.
*: These four ascites samples are from patients who had been treated with chemotherapy prior to collection for experimentation; the results using these four samples did not differ from chemotherapy-naïve samples.
n.a.: not available

Chapter 3

3 SNAIL ENHANCES THE METASTATIC POTENTIAL OF OVARIAN CANCER SPHEROIDS

3.1 Introduction

Ovarian cancer is the most lethal of the gynecologic malignancies in the developed world, existing predominantly as epithelial ovarian cancer (EOC) [1, 2]. Early detection significantly enhances survival of EOC patients; however, the overall prognosis remains poor since most patients (>75%) are initially diagnosed when the tumour has already metastasized beyond the primary site [3]. An accumulation of malignant fluid, or ascites, is commonly seen in the peritoneal cavity of these patients. Malignant EOC cells exist and survive in the ascites fluid as spheroid-like structures. EOC spheroids adhere and invade the peritoneum to seed metastatic tumour growth and are resistant to standard chemotherapeutics, providing unique therapeutic challenges for the treatment of EOC [4, 5].

Epithelial-mesenchymal transition (EMT) is an important mechanism responsible for the invasion and metastasis of many epithelial cancers [6]. EMT refers to the conversion of epithelial cells to fibroblast-like cells, and characterized by the loss of epithelial markers and acquisition of mesenchymal ones, and the enhancement of cell motility and invasion [7, 8]. In fact, EOC cells have the capacity to switch between epithelial and mesenchymal states during metastasis [4, 9].We have discovered that EOC patient ascites-derived cells naturally undergo a robust EMT response by simply aggregating into spheroids - the conduits of metastasis within ascites fluid [10]. Spheroid-induced EMT was characterized by the highest upregulation of Snail (*SNAI1*) amongst other factors and this was dependent on endogenous transforming growth factor-beta (TGFβ) signalling, which the most widely-studied initiator of EMT [11]. Indeed, we demonstrated that inhibiting endogenous TGFβ signalling in EOC cells ablates Snail expression and potently blocks EMT in EOC ascites-derived spheroid cells, compromising spheroid formation, viability, and cell motility and migration.

Snail serves a pivotal role in repressing epithelial genes during EMT including *CDH1,* which encodes E-cadherin [12]. Snail expression is a common indicator of poor prognosis in metastatic cancer, including ovarian, since tumours with elevated Snail expression are disproportionately more difficult to eradicate by current therapeutic treatments [12, 13]. In fact, Snail expression is positively correlated with the stage of human ovarian carcinoma, and it is significantly higher in metastatic lesions as compared with primary tumours [14]. Surprisingly, there is limited evidence regarding the functional role of Snail in EOC progression, especially in the context of three-dimensional spheroid pathobiology, a clinically-relevant metastasis model system [15]. Since we observed Snail to be the most highly upregulated mesenchymal marker induced upon spheroid formation and robustly reduced by blocking endogenous TGFβ signalling in EOC ascites-derived cells [10], we propose that Snail is the key downstream effector of TGFβ signalling in spheroid-induced EMT and is important for the survival and metastatic behaviour of EOC spheroids.

In this study, we explored Snail function in EOC spheroids using patient ascites-derived cells and ovarian cancer cell lines. Snail expression facilitated spheroid cell viability, and it was critical for the adhesion, migration, and invasion potential of ovarian cancer spheroid cells. Furthermore, using an *ex ovo* chickCAM assay, ectopic Snail expression in Hey ovarian cancer cell spheroids enhanced tumour-forming potential, invasion, and metastasis. These results expand our understanding of the function of Snail in ovarian cancer progression, highlighting the therapeutic potential for targeting Snail and the EMT process in late-stage ovarian cancer.

3.2 Materials and Methods

3.2.1 Cell culture

Ascites obtained from ovarian cancer patients (diagnosed with high-grade serous EOC) was used to generate primary cell cultures as described previously [16]. Ascites was mixed 1:1 with growth medium [MCDB105 (Sigma, St. Louis, MO) / M199 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Wisent, St. Bruno, Quebec, Canada) and 50 µg/mL penicillin-streptomycin. Experiments were performed

using cells between passages 3 and 5. All patient-derived cells were used in accordance with institutional human research ethics board approval (UWO HSREB 12668E). The iOvCa147-E2 cell line was generated from an ascites sample (EOC147) collected at our centre. OVCAR3 and OVCAR8 (purchased from ATCC, Manassas, VA) and Hey (gift from G. Mills, MD Anderson) cells were cultured in Dulbecco's Modified Eagle medium/F12 (Wisent) with 10% FBS (Wisent). Cells were grown in a 37°C humidified atmosphere of 95% air and 5% CO_2. Cell lines were authenticated by short tandem repeat analysis (Genetic Analysis Facility, The Hospital for Sick Children, Toronto, ON, Canada).

Adherent cells were maintained on tissue culture-treated polystyrene (Sarstedt, Newton, NC). Non-adherent cells (spheroids) were maintained for 72 h on Ultra-Low Attachment (ULA) cultureware (Corning, Corning, NY). For spheroid re-attachment assays, spheroids were removed from ULA cultureware and re-introduced to tissue culture-treated polystyrene for up to 72 h.

3.2.2 *SNAI1* (Snail) knockdown

Cells were plated at a density of 3×10^5 cells per 35 mm well in antibiotic-free media, and transfections were performed the next day using DharmaFECT1 as per manufacturer's protocol (Dharmacon; GE Healthcare, Mississauga, ON). Briefly, 1 µL of DharmaFECT1 was combined with 10 nM siRNA in 1 mL of media and incubated for 20 min, then added to each well. Media was replaced at 24 h with antibiotic-free growth media. Cells were incubated 72 h following transfection, then cells were trypsinized and seeded for adherent and spheroid cell viability assays. Human *SNAI1* siGENOME SMARTpool (M-010847-00) and non-targeting (control) siRNA was obtained from Dharmacon. Western blot analysis was performed on adherent cell lysates at 72 h post-transfection and 72 h post-seeding to ULA plates for spheroid lysates.

3.2.3 Ectopic *SNAI1* expression via retroviral transduction

Human Embryonic Kidney (HEK) 293T cells were seeded at 5×10^5 cells per 60 mm plate and maintained in DMEM with 5% FBS. Transfections were performed at 24 h using Lipofectamine® 2000 Reagent (Invitrogen) according to manufacturer's instructions.

Plasmids pMDG, pPSI, (gift from Dr. Mellissa Mann, Western U.) and pBABEpuro-SNAIL (Addgene) or pBABEpuro (Addgene) were co-transfected into HEK293T cells. Hey cells (approximately 80% confluent) were pre-treated for 45 min with 4 μg/mL polybrene (Sigma) prior to retroviral infection. Transfected HEK293T supernatant was clarified with a 0.45 micron syringe filter, diluted 1:1 in RPMI/5% FBS, and applied to Hey cells. At confluence, infected Hey cells were expanded and selected using 2 μg/mL puromycin to generate individual clones and populations.

3.2.4 Western blot analysis

Adherent and spheroid cells were washed once in cold phosphate-buffered saline (PBS), dissolved in lysis buffer [50 mM HEPES pH7.4, 150 mM NaCl, 10% glycerol, 1.5 mM MgCl2, 1mM EGTA, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 10 mM NaF, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 1x protease inhibitor cocktail (Roche, Laval, Quebec, Canada)], clarified by centrifugation (20 min at 15,000g), and quantified by Bradford analysis (Bio-Rad Laboratories, Mississauga, Ontario, Canada). Western blotting of protein lysates was performed as described previously [10]. Immunoreactive proteins were detected using enhanced chemiluminescence reagent (Luminata^{TM} Forte, Millipore) and imaged with the ChemiDoc MP System (BioRad, Mississauga, ON). Anti-Snail antibody (#3879) was purchased from Cell Signalling Technologies (Danvers, MA). Anti-actin antibody (A2066) was purchased from Sigma (Mississauga, ON).

3.2.5 Spheroid re-attachment assay

Macroscopically-visible spheroids formed from siNT or si*SNAI1*-transfected EOC ascites cells (n=7) after three days in suspension culture were transferred individually into wells of 48-well tissue culture-treated plates (minimum n=12 per sample). Re-attached spheroids were fixed and stained at 72 h (Hema-3 Stain kit; Fisher, Kalamazoo, MI), and images were captured using Leica DMI 4000B inverted microscope to quantify dispersion area (total area minus spheroid core) using *ImageJ* (NIH, Bethesda, MD).

3.2.6 Cell viability

Cells were seeded to either 24-well tissue culture plastic (1 x 10^4 cells/well) for adherent cultures, or ULA plates (5 x 10^4 cells/well) for spheroids. For si*SNAI1* experiments, spheroids were collected 72 h after seeding to ULA (6 days post-transfection), pelleted and left in media (100 μL), at which point CellTiter-Glo® reagent (Promega, Madison, WI) was added (1:1 v/v ratio). All samples were subjected to a freeze/thaw cycle prior to analysis of luminescence signal detected using a Wallac 1420 Victor 2 spectrophotometer (Perkin-Elmer, Waltham, MA). Under adherent conditions, cells were kept in original 24-well plates for 3 days and cell viability was assessed using alamarBlue® (Invitrogen, Burlington, ON) as per manufacturer's instructions. Fluorescence was quantified at 4 h using 560/590 nm excitation/emission filter settings on the Wallac spectrophotometer.

For measuring viability in HeyBABE and HeySNAIL spheroid cells, CyQUANT® NF (Invitrogen, Burlington, ON) assays were performed after 72 h in suspension culture. Briefly, spheroids were collected and pelleted, and medium was removed. After a single freeze-thaw, spheroids were resuspended in 1X Hank's balanced salt solution (HBSS), and 50 μL aliquots were mixed 1:1 with 2X dye binding solution in each microplate well; fluorescence was detected with the Wallac spectrophotometer using 485/530 nm filter settings.

3.2.7 **Immunofluorescence**

Confluent HeyBABE and HeySNAIL cells on glass coverslips were fixed in buffered 10% formalin, washed with PBS, and permeabilized with 0.1% Triton X-100/PBS. Overnight incubation with anti-Vimentin antibody (#5741, Cell signalling), or anti-Snail antibody (1:500 each) was followed by one-hour incubation with anti-rabbit fluorescein isothiocyanate-conjugated secondary antibody (1:500; Vector Laboratories) and a one-hour stain with Alexa Fluor® 568-conjugated phalloidin (A12380, Invitrogen). Coverslips were mounted in VectaShield mounting medium (Vector Laboratories) containing 4′,6-diamidino-2-phenylindole (1:1000; Sigma, St. Louis, MO). Fluorescence images were captured using an Olympus AX70 upright microscope and ImagePro image capture software.

3.2.8 Colony forming soft agar assay

HeyBABE and HeySNAIL cells were suspended in media (supplemented with 20% FBS) and mixed 1:1 with 1% agarose prior to seeding agarose pre-coated 6-well plates (at 2.5 x 10^4 cells/well). Media (with 10% FBS) was added to each well, and replaced every 48 h for 2 weeks. Images were captured (six fields of view per well) using a Leica DMI 4000B inverted microscope, and colonies were counted using *ImageJ* software.

3.2.9 Scratch-wound assay

Confluent adherent cultures of HeyBABE and HeySNAIL cells were scratched with a P1000 tip and washed twice with PBS to remove detached cells, and media was replaced. Images were captured using a Leica DMI 4000B inverted microscope at initiation (0 h) and at 4 and 10 h post-wounding, and cell motility was quantified using *ImageJ* software.

3.2.10 Adhesion assay

HeyBABE and HeySNAIL spheroids were transferred to tissue culture plastic, collagen-coated plates (354402, Corning), or fibronectin-coated plates (152034, Thermo Scientific Nalgene), and incubated for 30 min. Re-attached spheroids were fixed and HEMA3-stained after 30 min, and quantified. Representative images were captured using using a Zeiss Axiozoom.V16 stereo macroscope and ZEN software (ZEISS).

3.2.11 Transwell migration and invasion assay

EOC ascites cells were formed from siNT or si*SNAI1* transfection cells seeded at 5 x 10^4 cells per well in 24-well ULA plates. Spheroids formed from HeyBABE and HeySNAIL cells were seeded at 2.5 x 10^4 cells per well in 24-well ULA plates. After 3 days, PBS-washed spheroids were transferred individually to 100 µL of reduced serum media (0.5% FBS) and added to the top chamber of a Transwell (6.5 mm in diameter; 8 µm pore size) (Costar®), with 600 µL of medium containing 10% FBS in the bottom chamber. Transwell membranes were fixed and stained with Hema-3 Stain kit at 12 h and non-migrated cells were removed using a cotton swab. Migrated cells were imaged with a Leica

DMI 4000B inverted microscope (five fields/membrane) and counted with *ImageJ* software.

Transwell membranes were coated with either rat tail collagen I (A1048301, Gibco® Life Technologies) or human fibronectin (PHE0023, Gibco® Life Technologies) matrices. Briefly, stock solutions for each were diluted to 50 µg/mL, and 50 µL was added to the apical chamber and incubated for 2 h at 37°C. The remaining solution was aspirated dried for 30 min, then media was added to the apical and basolateral compartments for 30 min at 37°C. HeyBABE and HeySNAIL spheroid cell invasion was performed as described above in migration assays but were allowed to incubate for 18 h.

3.2.12 Chick embryo chorioallantoic membrane (CAM) assay

Adherent HeySNAIL and HeyBABE cells were labelled with Molecular Probes™ Lipophilic Tracer DiO at 1:500 for 2 h. Cells were washed twice with PBS, trypsinized, pelleted, and re-suspended in fresh media (supplemented with 10% FBS) for subsequent seeding into 6-well ULA plates at 5 x 10^4 cells per well to form spheroids. Three days later, spheroids were used for chick-CAM assays which were prepared as previously described [17-21]. Using embryonic day-10 chicks, 6 DiO-labelled spheroids in 20 µL of serum-free media were implanted between branching vessels on the CAM and incubated in a humidified 37°C stationary hatcher for 7 d. Brightfield and fluorescent images of tumour were captured using the Zeiss Axiozoom.V16 stereo microscope and ZEN software. The total number of chicks were n=48 for HeySNAIL and n=52 for HeyBABE spheroid over 6 independent experiments.

Tumours were harvested for histology (H&E and human anti-Ki-67 immunostaining) and slides were imaged using Aperio ScanScope slide scanner (Leica Biosystems). Livers were harvested and genomic DNA was extracted using a QIAamp Fast DNA Stool mini kit (51604, Qiagen), and 15 ng of DNA was used for quantitative PCR using Brilliant SYBR Green QPCR Master Mix (Agilent Technologies/Stratagene) and a QuantStudio3 thermocycler (Thermo Fisher Scientific). Human-specific primers for *Alu* sequences [forward: 5'- ACG CCT GTA ATC CCA GCA CTT – 3'; reverse: 5'- TCG CCC AGG CTG GAG TGC A -3'] (Sigma) were used to detect tumour cells and chicken

Gapdh [forward: 5'- AGA GAA AGG TCG CCT GGT GGA TCG -3'; reverse: 5'- GGT GAG GAC AAG CAG TGA GGA ACG -3'] served as an internal control for RNA input with quantification using the $2^{\Delta Ct}$ method [22].

3.2.13 Statistical analysis

Data were expressed as mean ± SEM. Statistical analyses were performed using a two-tailed Student's *t*-test or one-way analysis of variation (ANOVA) with Tukey's Multiple Comparison test.

3.3 Results

3.3.1 *SNAI1* knockdown in EOC ascites-derived cells reduces spheroid cell migration

We have demonstrated previously that Snail is consistently the most up-regulated EMT marker in EOC spheroids [10]. In addition, among the classical transcriptional repressors known to be involved in mediating EMT in cancer, *SNAI1* (encoding Snail) possesses the most frequent copy-number alterations (as evidenced by amplifications and overexpression) among serous ovarian carcinomas in the Cancer Genome Atlas dataset and the 50 established cell lines from the Cancer Cell Line Encyclopedia dataset [23, 24] (Figure 3.1). It should also be noted, that there are no deletions in *SNAI1* identified in either of these two large datasets, indicating a potential selective pressure to retain Snail expression and function in ovarian cancer.

To initiate our analysis of Snail function in EOC spheroids, we utilized an siRNA-mediated knockdown approach to reduce Snail expression. First, we assayed for the expression of Snail protein in several cell lines cultured as adherent cells or spheroids. Consistent with our previous observations in EOC ascites-derived cells [10], we observed Snail up-regulation in spheroids generated from Ovcar8, Ovcar3, and iOvCa147-E2 ovarian cancer cell lines (Figure 3.2A). Using these cell lines, transient Snail knockdown was performed in adherent culture and spheroids (Figure 3.2B). In day-3 spheroids, si*SNAI1*-transfected cells formed smaller, loosely-aggregated spheroids when compared

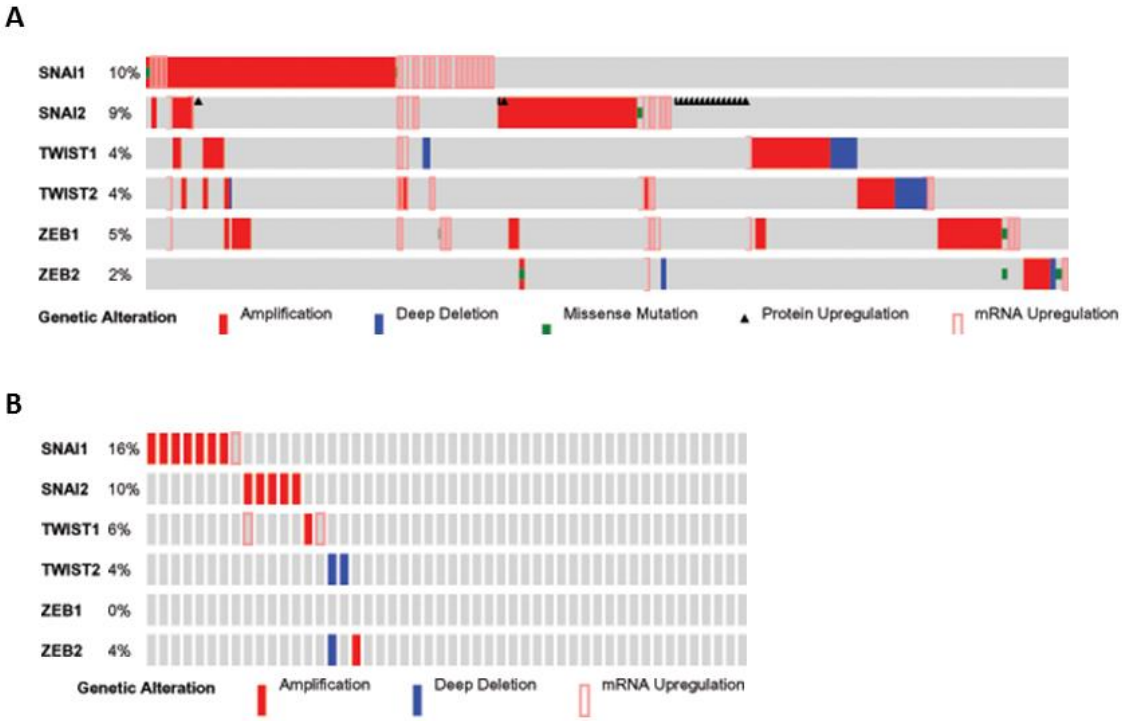


Figure 3.1: Oncoprint analysis of SNAI1 and its EMT-related transcriptional repressor genes in high-grade serous ovarian carcinoma.
Putative copy number alterations from GISTIC, gene mutation, and mRNA expression (RNA seq V2 RSEM) for *SNAI1*, *SNAI2*, *TWIST1*, *TWIST2*, *ZEB1* and *ZEB2* were assessed in ovarian serous carcinomas using cBioPortal (TCGA provisional dataset, 597 cases). Only samples with alterations are shown.

with siNT controls in Ovcar3 and iOvCa147-E2 cell lines (Figure 3.2C). Snail knockdown did not affect the ability of Ovcar8 cells to form multicellular aggregates. Reduced Snail expression had no effect on the viability of adherent cells in all three cell lines, but resulted in decreased cell viability in Ovcar3 and iOvCa147-E2 spheroids (Figure 3.2D), which correlated with spheroid morphology. Thus, Snail may be necessary for normal spheroid formation and cell viability in these structures, at least in a subset of ovarian cancer cell lines.

Transient *SNAI1* knockdown in several EOC ascites-derived cells (n=4) was achieved in adherent monolayer culture, and sustained for 3 days in suspension culture during spheroid formation (Figure 3.3A). Snail knockdown had variable effects on EOC spheroid formation: either *siSNAI1* had no effect on spheroid structure (data not shown), or si*SNAI1*-transfection yielded loosely-aggregated spheroids as compared with siNT controls (Figure 3.3B). Snail knockdown had no effect on the viability of adherent EOC ascites cells, but caused a significant yet modest reduction in cell viability of EOC spheroids among all samples tested (Figure 3.3C). These data indicate that Snail up-regulation in EOC ascites-derived spheroids promotes an EMT phenotype, but may not be a critical factor for maintaining cell viability in these structures.

We previously demonstrated that inhibition of endogenous TGFβ signalling and resultant EMT in EOC spheroids decreases cell motility of dispersing cells upon spheroid re-attachment, as well as spheroid cell migration [10]. Herein, we sought to investigate whether Snail specifically controls cell motility and migration during EOC spheroid re-attachment and dispersion. To assess cell motility, spheroids formed from EOC ascites-derived cells transfected with si*SNAI1* or siNT control were seeded individually for re-attachment to standard tissue culture plastic (n=7). At 72 h, no differences in dispersion area were observed between siNT and si*SNAI1* re-attached spheroids (Figure 3.4). However, we observed that *SNAI1* knockdown in EOC ascites-derived spheroids significantly reduced cell migration in Transwell migration assays as compared with siNT controls (Figure 3.5D and E). Thus, up-regulated Snail expression in EOC ascites-derived spheroids is required for promoting cell migration during re-attachment.

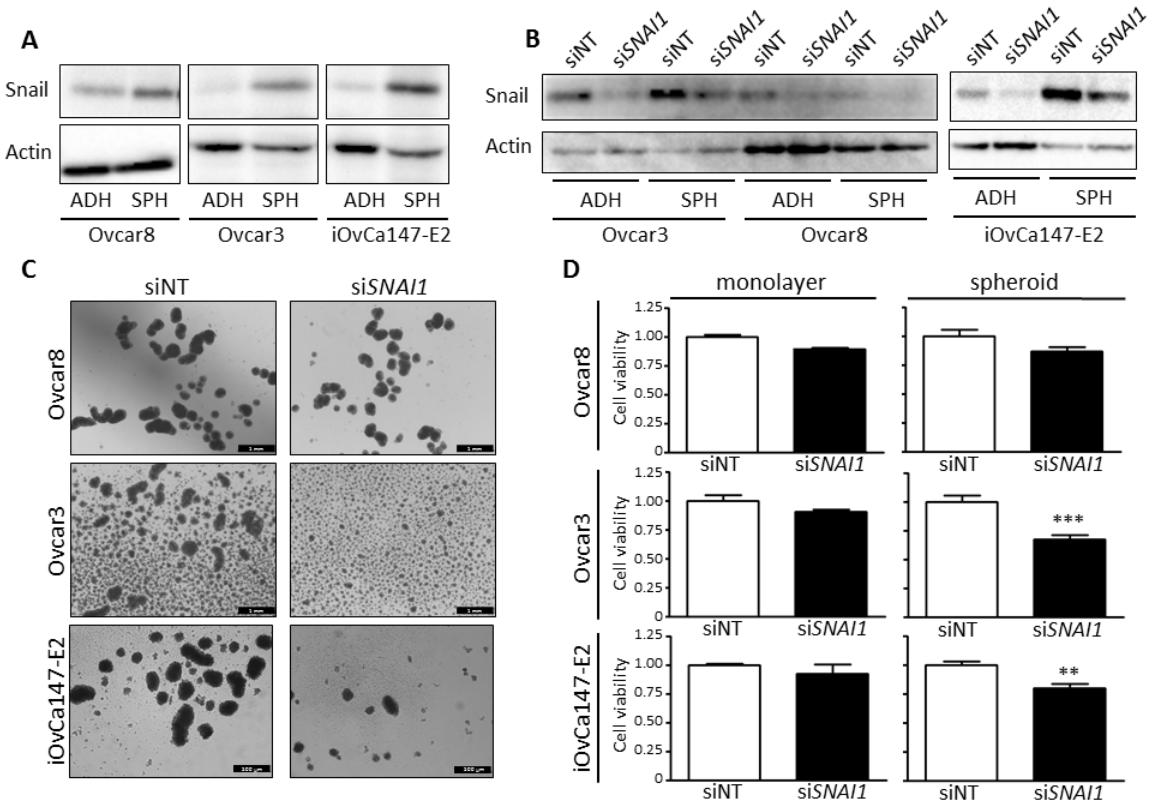


Figure 3.2: SNAI1 knockdown in Ovcar3 and iOvCa147-E2 ovarian cancer cell lines reduces spheroid cell viability.

(A) Ovcar8, Ovcar3, and iOvCa147-E2 cell lines express increased Snail protein in spheroids compared with adherent cells as determined by Western blotting. Actin served as a loading control. (B) *SNAI1* knockdown in transfected adherent and spheroid ovarian cancer cell lines; siNT served as a negative control and actin was used as a loading control. (C) *SNAI1* knockdown in Ovcar3 and iOvCa147-E2 cells reduces spheroid formation as evidenced by increased cell debris; no effect was observed with Ovcar8 spheroids. (D) Cell viability in monolayer cells and spheroids using alamarBlue assay and CellTiter-Glo, respectively (**, $p<0.01$; ***, $p<0.001$ as determined by Student's *t*-test).

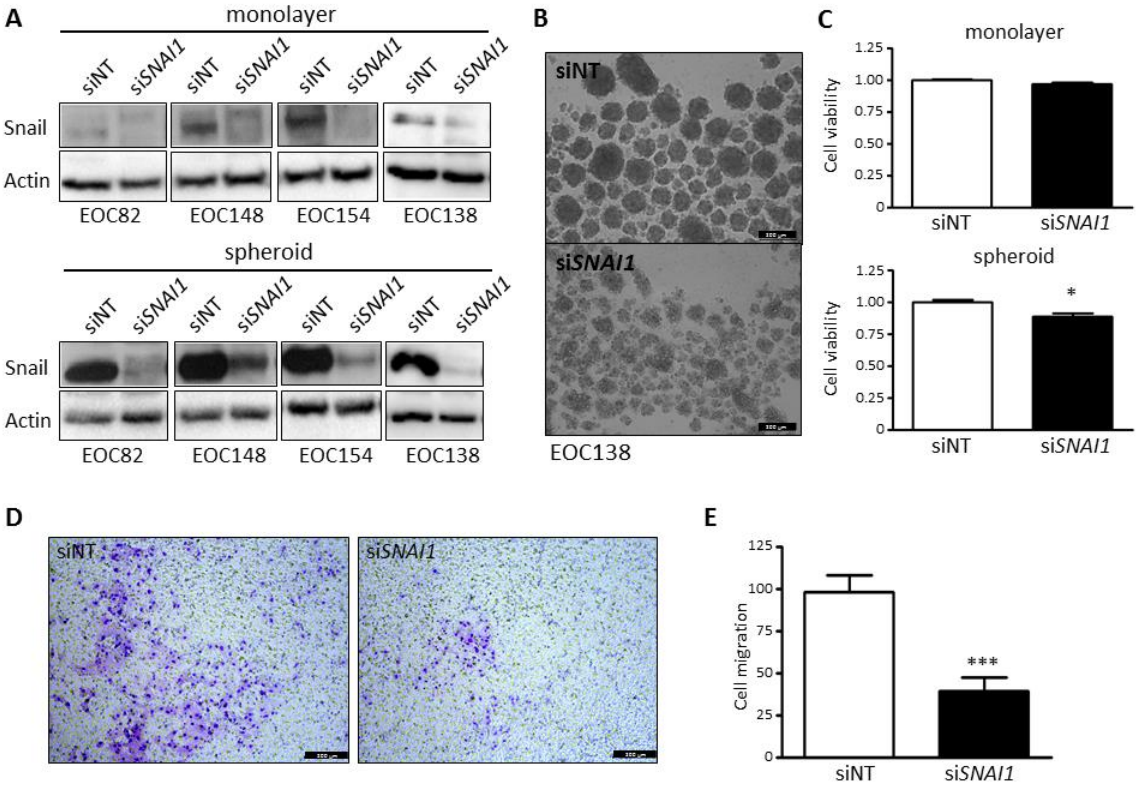


Figure 3.3: SNAI1 knockdown in EOC ascites-derived cells reduces spheroid cell viability and migration.

(A) Transient *SNAI1* knockdown in monolayer and spheroid cells using four EOC patient ascites-derived samples with control non-targeted siRNA (siNT) as determined by Western blotting. Actin served as a loading control. (B) Phase contrast images of spheroids from representative ascites-derived cell transfections demonstrating variable *SNAI1* knockdown effects on spheroid structure. (C) Cell viability of siRNA-transfected EOC ascites-derived monolayer cells and spheroids using alamarBlue assay and CellTiter-Glo, respectively. (n=4; *, $p<0.05$ as determined by Student's *t*-test). (D) Spheroids formed from EOC ascites-derived cells transfected with si*SNAI1* or siNT control were seeded to Transwell inserts with migration towards 10% FBS for 12 h; representative images of migrated stained cells are shown. (E) EOC ascites-derived si*SNAI1*-transfected spheroid cells have decreased cell migration as quantified among five fields per image (n=3; ***, $p<0.001$ as determined by Student's *t*-test).

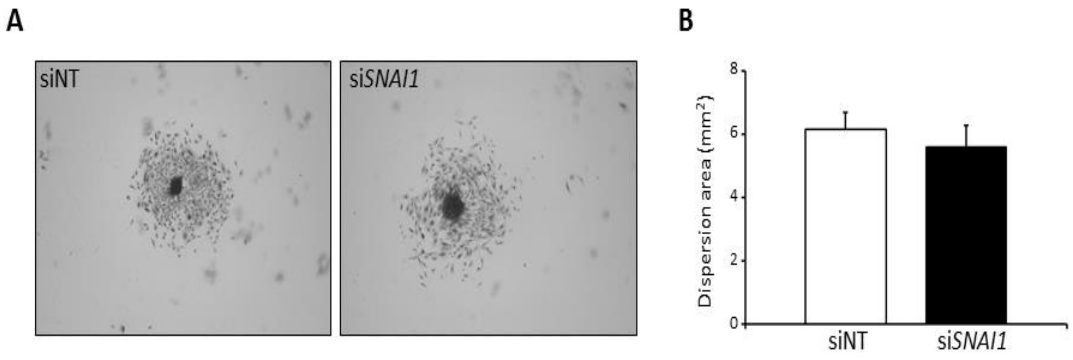


Figure 3.4: SNAI1 knockdown in EOC ascites-derived cells does not affect spheroid re-attachment.
(A) Spheroids formed from EOC ascites-derived cells transfected with si*SNAI1* or siNT control were seeded individually for reattachment to tissue culture plastic; representative images are shown. (B) Area of cell dispersion was quantified at 72 h post-reattachment using ImageJ. No difference in dispersion area was observed between si*SNAI1* and siNT reattached spheroids (n=7; Student's *t*-test).

3.3.2 Ectopic Snail expression in Hey cells induces a robust mesenchymal phenotype

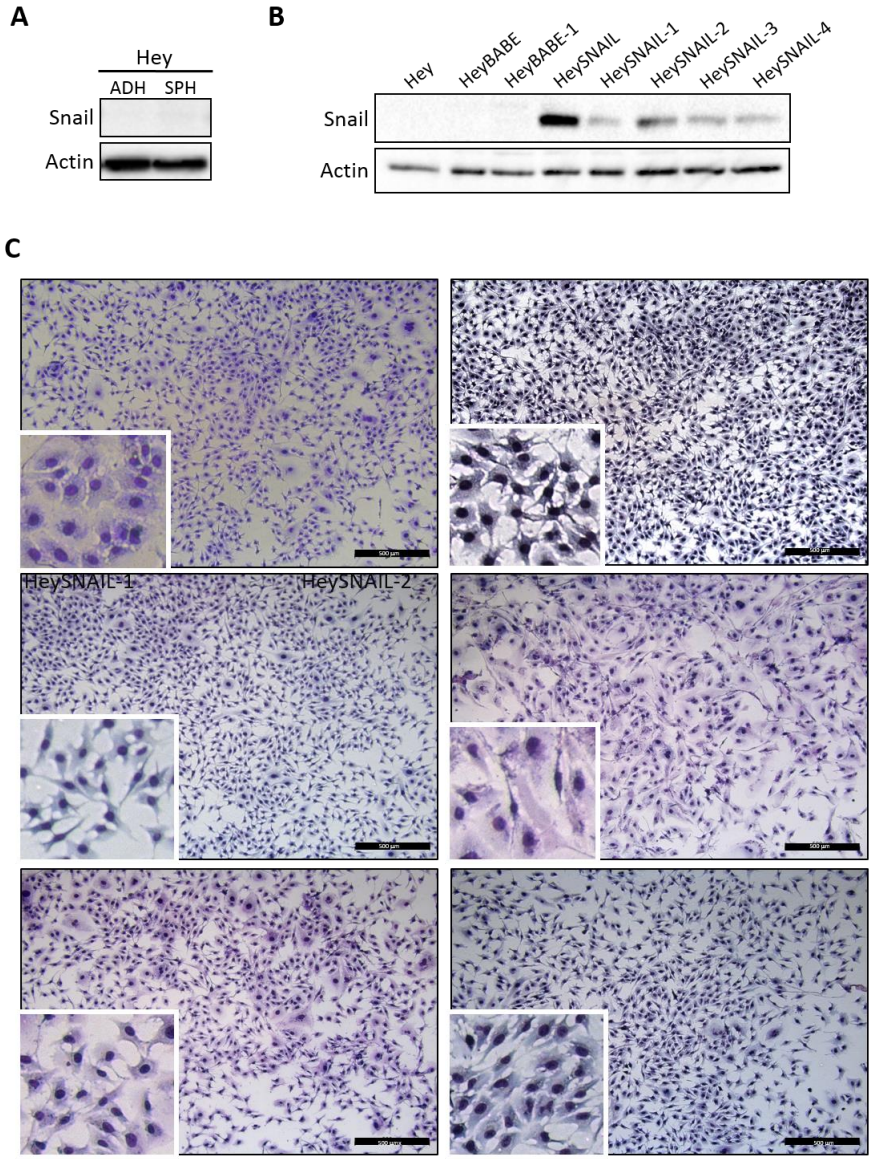
Assessment of Snail expression in several ovarian cancer cell lines showed that Hey cells lack Snail protein expression and it is not induced in spheroids (Figure 3.5A). Thus, we ectopically expressed Snail in Hey cells to examine the functional consequences of ectopic expression. Retroviral transduction was followed to generate populations (HeySNAIL) as well as several individual clones (HeySNAIL-1, -2, -3, and -4); control Hey cell populations (HeyBABE) and as well as clones (HeyBABE-1) (Figure 3.5B; Figure 3.6).

Ectopic Snail expression in Hey cells induced a robust and sustained mesenchymal phenotype in adherent HeySNAIL populations and clones as compared with control cells (Figure 3.5C and D). Snail-expressing Hey cells had a more fibroblastic spindle-shaped morphology (Figure 3.5C) with intense stress fiber formation and enhanced vimentin staining (Figure 3.5D), indicating an induced mesenchymal phenotype as compared with controls.

To further characterize the malignant potential of Snail-expressing Hey cells, we assayed for cell proliferation, anchorage-independent growth and cell motility. HeySNAIL populations and clones had reduced cell proliferation relative to control cells in adherent culture (Figure 3.7A). To assess anchorage-independent growth, HeySNAIL cells and controls were seeded in soft agar; over 14 d, Snail expression enhanced Hey cells colony formation as compared with control cells (Figure 3.7B). Scratch wound assays, however, revealed no significant differences in cell motility of Hey cells with or without Snail expression (Figure 3.7C and D).

3.3.3 HeySNAIL spheroid cells have enhanced adhesion, invasion and migration potential

Since we have observed disrupted spheroid formation and reduced EOC spheroid cell migration by TGFβ signalling inhibition [10] and Snail knockdown (Figure 3.3D and E), we assessed spheroid formation and migration capacity of HeySNAIL cells compared


A
Hey
ADH
SPH
Snail
Actin
B
Hey
HeyBABE
HeyBABE-1
HeySNAIL
HeySNAIL-1
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HeySNAIL-3
HeySNAIL-4
Snail
Actin
C
HeySNAIL-1
HeySNAIL-2

D

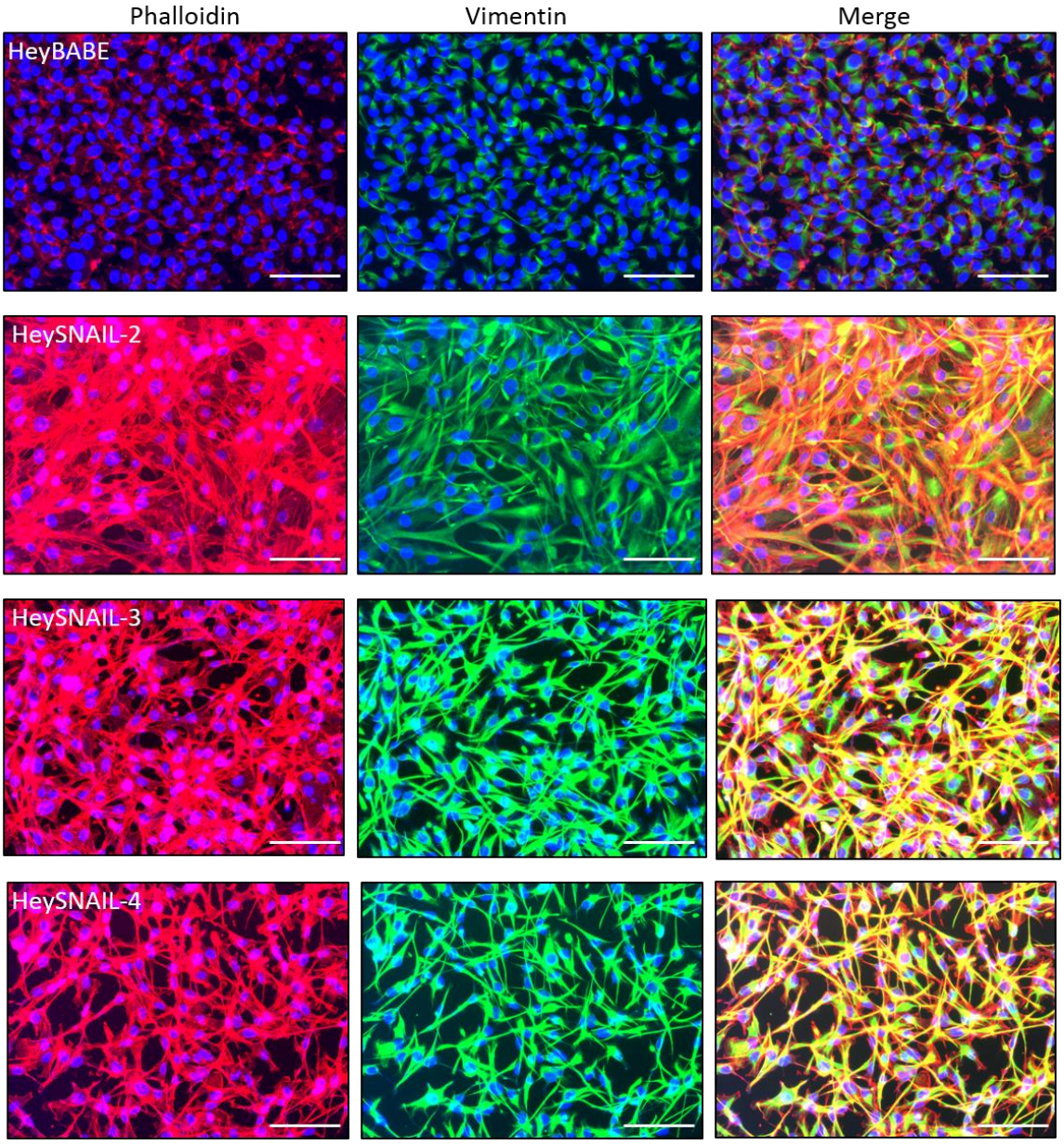

Phalloidin
Vimentin
Merge
HeyBABE
HeySNAIL-2
HeySNAIL-3
HeySNAIL-4

Figure 3.5: Ectopic Snail expression in Hey cells induces a robust mesenchymal phenotype.
(A) Hey cells do not express endogenous Snail protein whether in adherent or spheroid culture. (B) Snail protein expression as determined by Western blotting in retrovirally-transduced Hey cells. HeySNAIL and HeyBABE controls represent mixed populations, whereas HeySNAIL-1, -2, -3 and -4 and HeyBABE-1 represent individual clones. Actin was used as a loading control. (C) Brightfield images of Hema3-stained HeySNAIL cells and clones exhibit an enhanced mesenchymal or spindle-shaped morphology as compared with HeyBABE control cells. (D) Fluorescence images of HeySNAIL-2, -3 and -4 cell clones show enhanced stress fiber formation (rhodamine-conjugated phalloidin) and vimentin expression as compared with HeyBABE control cells. *Scale bar=100μM*

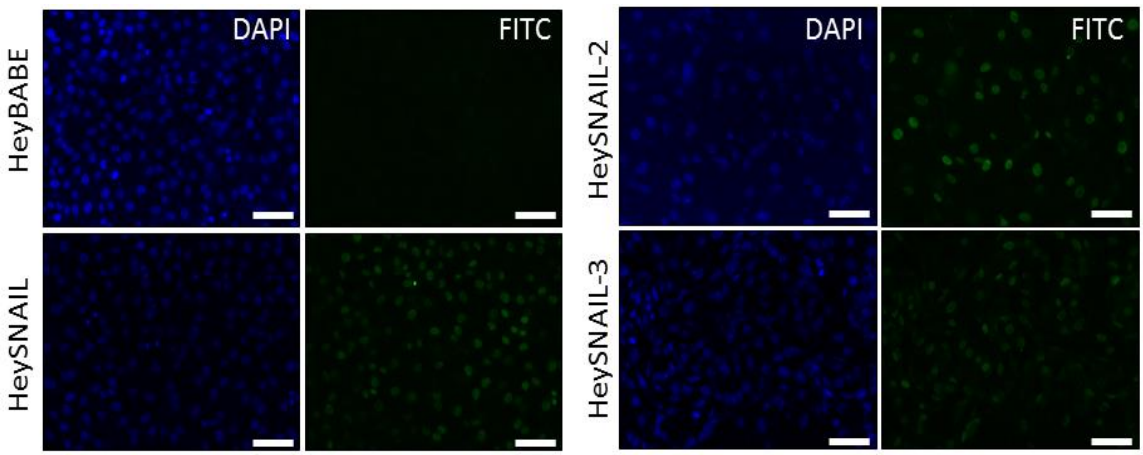


Figure 3.6: Immunofluorescence microscopy of Snail in HeySNAIL cell populations and clones.
Nuclear Snail immune-staining is present in HeySNAIL cell populations and clones, and absent in HeyBABE population cells.

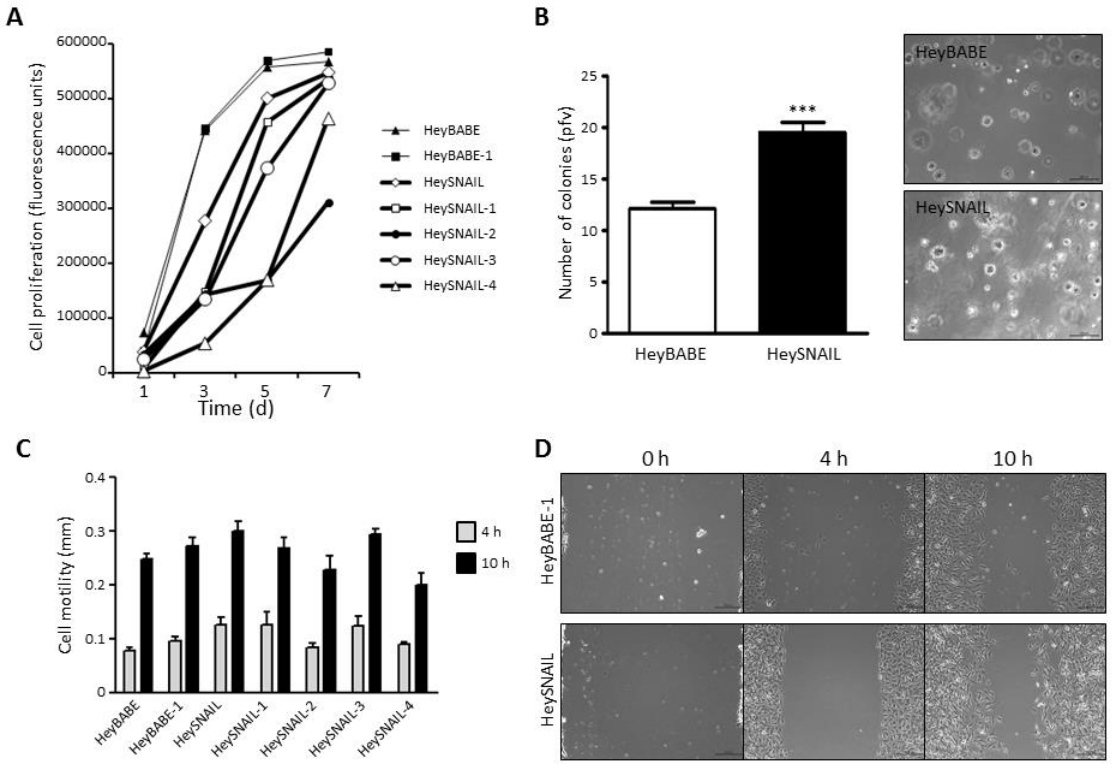


Figure 3.7: HeySNAIL cells have reduced proliferation but enhanced growth in soft agar.
(A) Proliferation of HeyBABE and HeySNAIL cell populations and clones was measured by alamarBlue assay at days 1, 3, 5, and 7 after seeding. (B) Colonies of HeyBABE and HeySNAIL cells grown in soft agar over 14 days were imaged and quantified; representative images are shown. (C) Scratch wound assays were performed using HeyBABE and HeySNAIL cell populations and clones and cell motility was measured at 4 and 10 h post-wounding. (D) Representative phase contrast images of scratch wound assays using HeyBABE-1 and HeySNAIL cell populations. Images were captured at initiation of the wound (0 h) and at 4 and 10 h post-wounding.

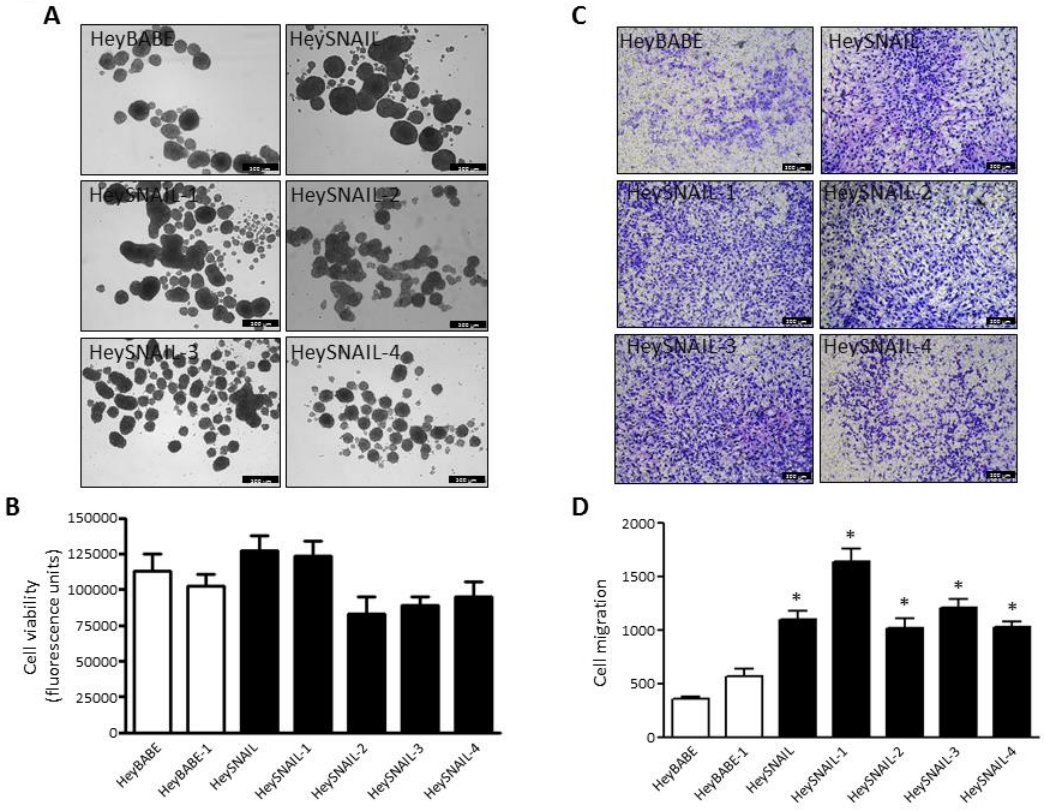


Figure 3.8: HeySNAIL spheroid cells have enhanced Transwell migration.
(A) HeyBABE and HeySNAIL cell populations and clones were seeded into 24-well ULA plates. Representative images were captured at day-3 in suspension. (B) Spheroid cell viability was measured using CyQUANT assay. (C) Spheroids formed from HeyBABE and HeySNAIL cell populations and clones were seeded to Transwell inserts containing 0.5% FBS and allowed to attach and migrate towards 10% FBS for 12 h; representative images of migrated stained cells are shown. (D) Number of migrated cells was quantified and averaged amongst five different fields per sample (*, $p<0.05$ as determined by Student's *t*-test).

with control cells lacking Snail expression. Snail-expressing Hey cells formed spheroids of similar morphology as compared with controls (Figure 3.8A). Indeed, there were no significant differences in cell viability among spheroids formed from all HeySNAIL cells and controls (Figure 3.8B). Although there were no effects on spheroid formation, HeySNAIL spheroids showed significantly enhanced cell migration capacity through Transwell membranes as compared with control spheroids (Figure 3.8C and D).

These results were further explored by adhesion and invasion assays performed using collagen and fibronectin matrices, which are established substrates for EOC cell attachment [25, 26]. Hey spheroids expressing Snail displayed significantly enhanced adhesion to standard tissue-culture plastic (Figure 3.9A and Figure 3.10A) and fibronectin-coated wells (Figure 3.9B and Figure 3.10B) as compared with control spheroids. There was a trend towards increased cell adhesion to collagen for HeySNAIL spheroids (Figure 3.9C and Figure 3.10C). In addition, Snail-expressing Hey spheroids exhibited enhanced invasion through both collagen (Figure 3.9D and E; Figure 3.11A) and fibronectin (Figure 3.9F and G; Figure 3.11B) matrices.

3.3.4 HeySNAIL spheroids have enhanced tumour-forming potential in an *ex ovo* chick CAM assay

We wished to rapidly assess whether ectopic Snail expression in Hey spheroid cells would enhance their ability to attach and form secondary tumours akin to ovarian cancer *trans*-coelomic metastasis mechanisms. To this end, we investigated spheroid attachment and tumour formation using an *ex ovo* chick embryo chorioallantoic membrane (CAM) assay. Using the *ex ovo* chick CAM as a xenograft model facilitates the rapid and direct visualization of tumour growth over a duration of only 7 d [17, 18]. HeySNAIL and control cells were first DiO-labelled prior to spheroid formation to track tumour cell growth and monitor invasion on the CAM surface; we confirmed DiO-label retention in spheroids over 7 d in culture (Figure 3.12A). After 7 d post-implantation of spheroids on the CAM (6 spheroids were xenografted in a single location per embryo), we observed a significant increase in xenograft tumour size generated by HeySNAIL spheroids (n=48 embryos) as

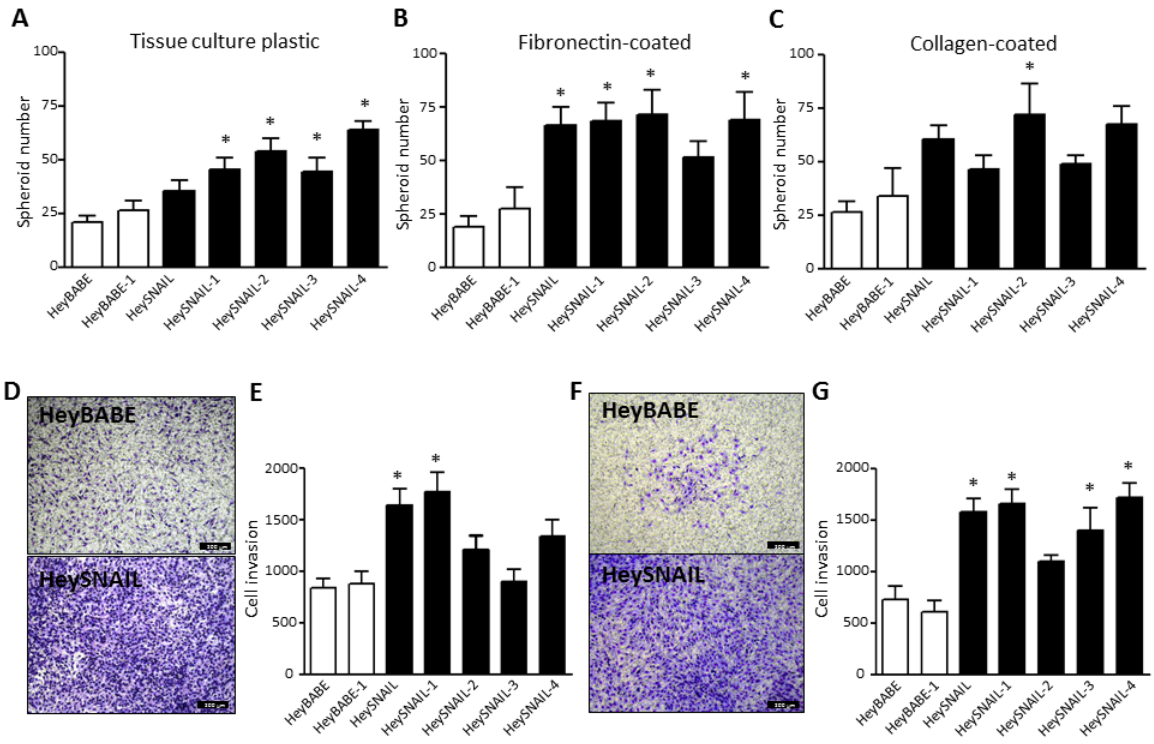


Figure 3.9: HeySNAIL spheroid cells display enhanced adhesion and invasion. (A, B, C) Spheroids formed from HeyBABE and HeySNAIL cell populations and clones were transferred to standard tissue culture plastic plates (A), fibronectin-coated plates (B), or collagen-coated plates (C). Re-attached spheroids were quantified at 30 min post-reattachment. (D, E) Spheroids formed from HeyBABE and HeySNAIL cell populations and clones were seeded to collagen-coated Transwell inserts and allowed to migrate towards 10% FBS for 18 h. Representative images are shown (D) and quantification averaged amongst five fields per sample (E). (F, G) Transwell invasion assays using fibronectin-coated inserts with representative images (F) and quantification averaged amongst five different fields per sample (G). (*, $p<0.05$ as determined by Student's *t*-test).

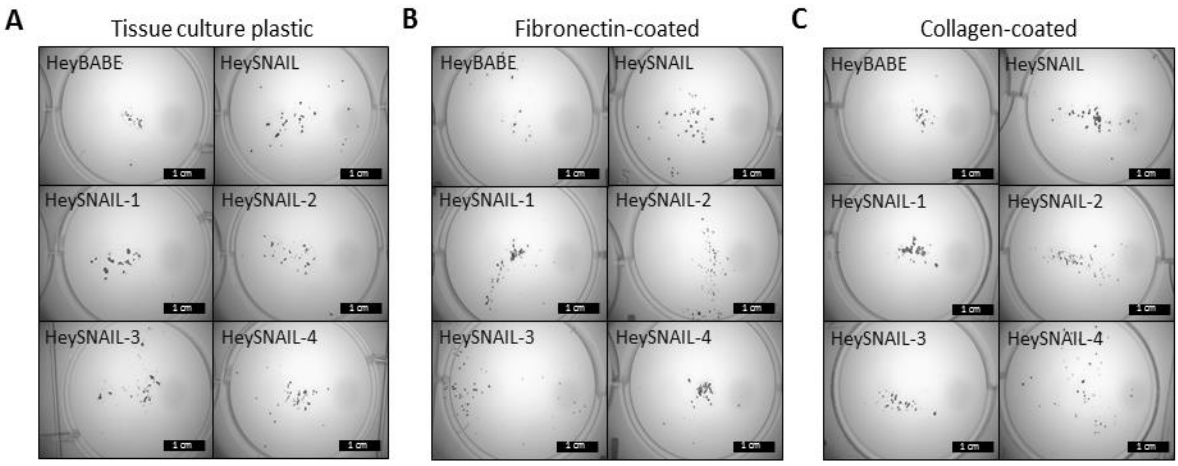


Figure 3.10: HeySNAIL spheroid cells have enhanced adhesion.
(A, B, C) Spheroids formed HeyBABE and HeySNAIL cell populations and clones were transferred to standard tissue culture plastic plates (A), fibronectin-coated plates (B), or collagen-coated plates (C). Re-attached spheroids were fixed and HEMA3-stained at 30 min post-reattachment; representative images are shown.

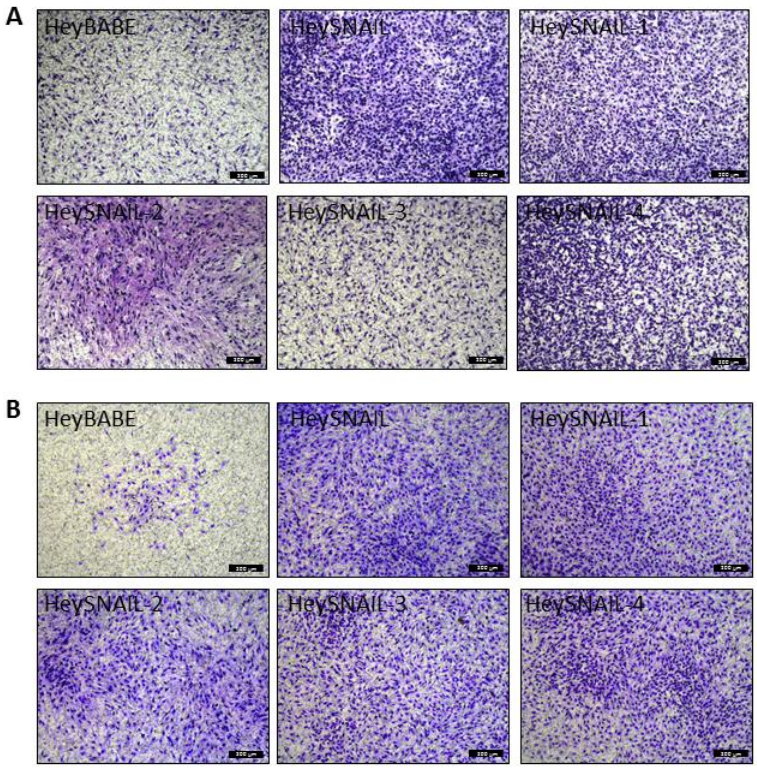
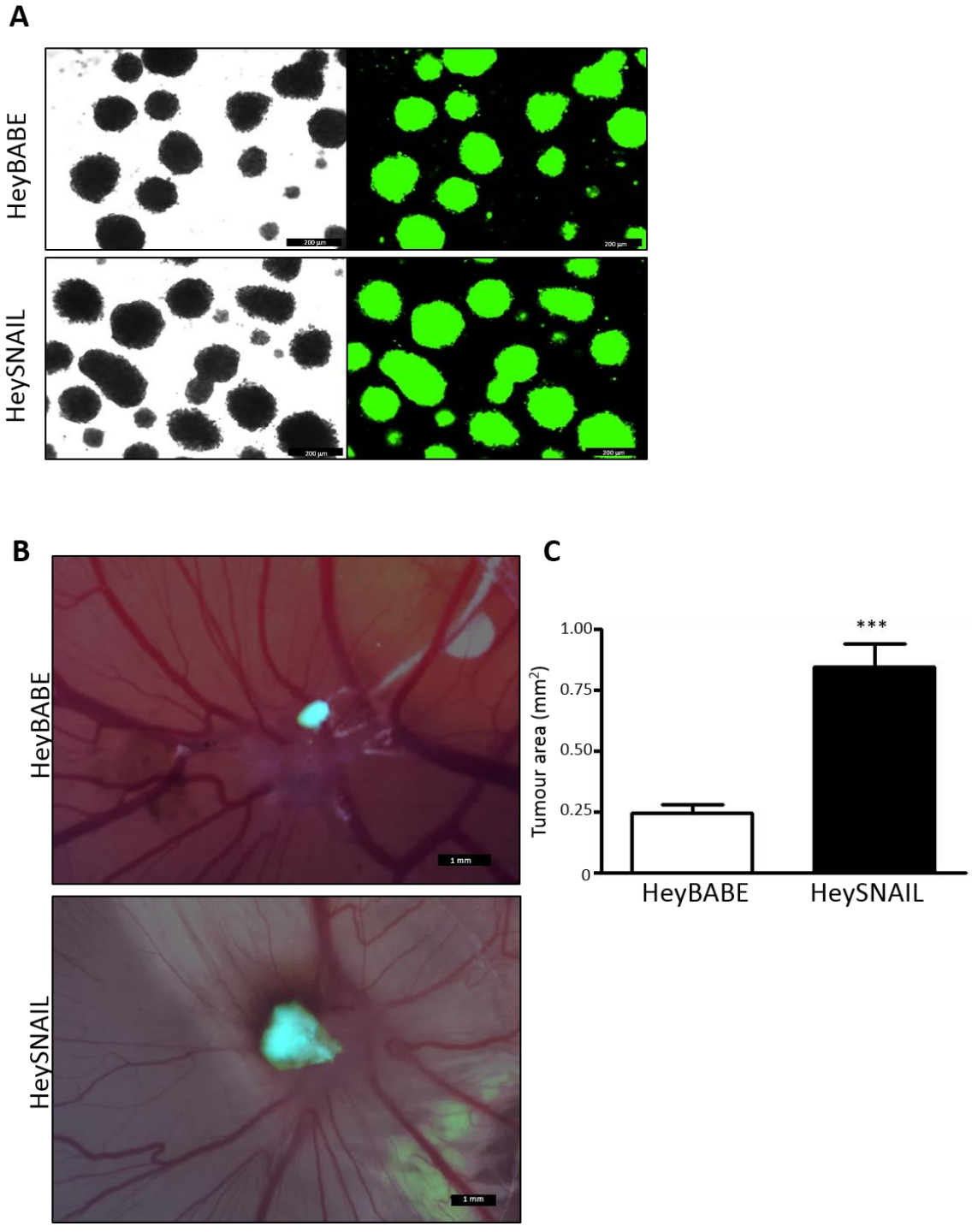


Figure 3.11: HeySNAIL spheroid cells have enhanced invasion.
(A) Spheroids formed from HeyBABE and HeySNAIL cell clones were seeded to collagen-coated Transwell inserts and allowed to migrate towards 10% FBS for 18 h. Representative images of migrated HEMA3-stained cells are shown. (B) Spheroids formed from HeyBABE and HeySNAIL cell clones were seeded to fibronectin-coated Transwell inserts and allowed to migrate towards 10% FBS for 18 h. Representative images of migrated HEMA3-stained cells are shown.

compared with control spheroids (n=52 embryos)(Figure 3.12B and C). Representative samples revealed larger, well-defined and apparently more invasive tumour xenografts resulting from HeySNAIL spheroids (Figure 3.12B). Fifteen specimens from each group were assessed further by histologic analysis. In sections with discernible tumour cells, the HeySNAIL group consisted of 8 samples showing cancer cell invasion into the CAM stroma and 5 samples with no invasion, where tumour cells remained on the CAM surface. In contrast, HeyBABE control spheroids showed only 3 samples with CAM invasion and 11 with surface tumour cells (data not shown). Human anti-Ki-67 immunostaining confirmed presence of xenografted tumour cells, as well as their proliferative potential (Figure 3.12D). Since the HeySNAIL spheroid xenografts appeared to have increased invasion into the CAM, metastatic capacity was assessed by liver dissection and quantitative PCR using isolated genomic DNA from a subset of embryos (n=8 for each group). Using primers for human *Alu* repeat DNA sequences [18], we observed a trend towards greater metastasis to the liver in the HeySNAIL group relative to the HeyBABE controls (p=0.052)(Figure 3.12E).

Taken together, our results strongly suggest that Snail expression in ovarian cancer spheroids potentiates adhesion and invasion of these multicellular structures, thereby resulting in an enhanced capacity to seed secondary tumour formation.


A
HeyBABE
HeySNAIL
200 µm
B
HeyBABE
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1 mm
C
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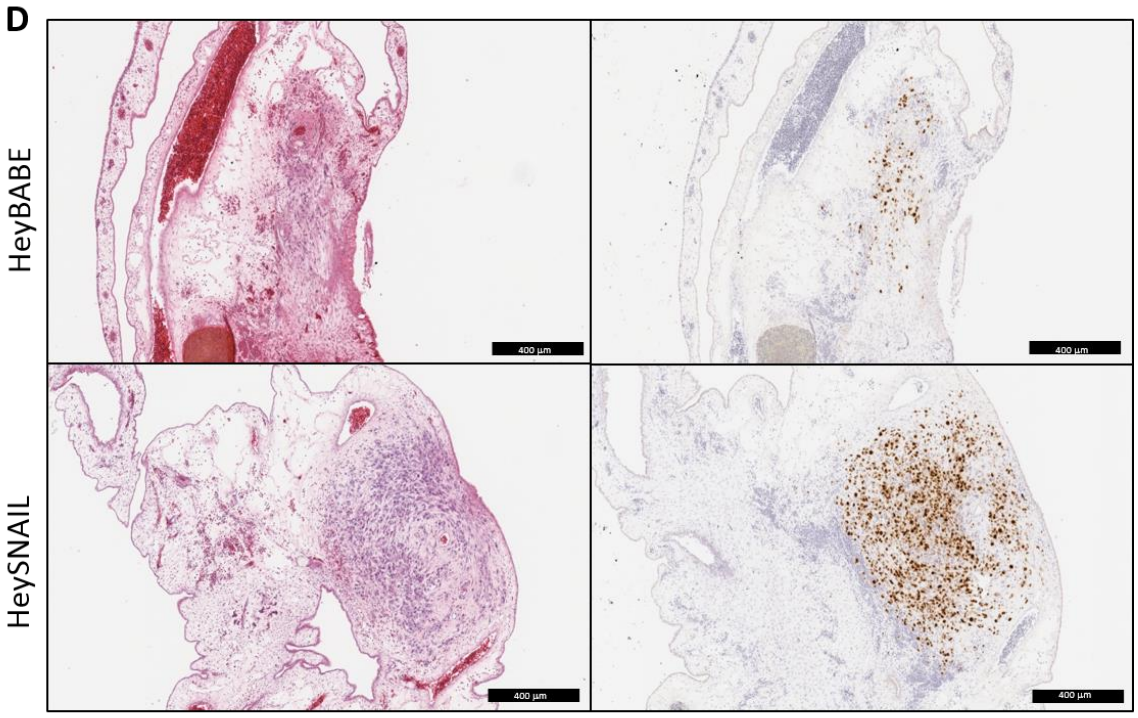
D
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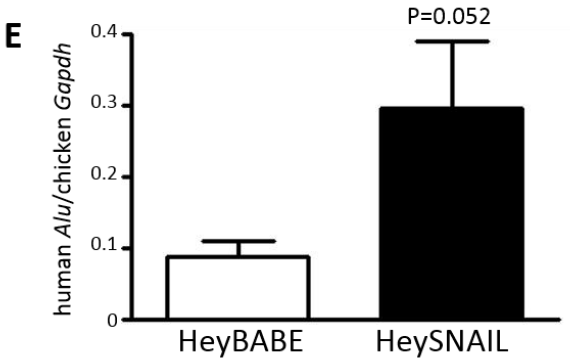
E
P=0.052
human Alu/chicken Gapdh
0.4
0.3
0.2
0.1
0
HeyBABE
HeySNAIL

Figure 3.12: HeySNAIL spheroids have enhanced tumour-forming potential in an ex vivo chick CAM assay.
(A) Brightfield and fluorescence microscopy images of day-7 spheroids formed from HeyBABE and HeySNAIL cell populations stained with DiO prior to spheroid formation. (B) DiO-labelled HeyBABE and HeySNAIL spheroids were inoculated onto the chick CAM surface (six spheroids per CAM) and imaged after 7 d incubation. Representative brightfield/fluorescence merged images of HeyBABE and HeySNAIL tumours are shown. HeySNAIL cells formed much larger tumours. (C) Tumour area was measured using ImageJ from six independent experiments (n=48, HeySNAIL; n=52, HeyBABE). (***, $p<0.001$ as determined by Student's t-test). (D) Representative H&E (left) and human anti-Ki-67 immunostaining (brown-stained nuclei; right) for HeyBABE and HeySNAIL tumours invading the CAM tissue. (E) Genomic DNA was extracted from the liver of HeyBABE and HeySNAIL groups (n=8 per group) to perform qPCR with primers specific for human Alu sequences and normalized to chicken Gapdh. There was a strong trend towards higher liver metastasis in the HeySNAIL group compared to controls ($p=0.0502$ as determined by Student's t-test).

3.4 Discussion

Spheroids serve as the vehicle for dissemination of malignant EOC cells in the peritoneal cavity of patients with late-stage disease, protecting cells from anoikis induced stress in the extracellular compartment [4, 15]. We have recently observed that epithelial ovarian cancer cells induce an endogenous EMT response upon spheroid formation that is regulated by endogenous TGFβ signalling and characterized by the robust upregulation of Snail in EOC spheroids [10]. Since Snail upregulation is dramatically reduced by TGFβ signalling inhibition, herein we sought to investigate the role of Snail in directly mediating the malignant potential of EOC spheroids and metastasis of this devastating disease. Via *SNAI1* knockdown experiments, we show that Snail is required to promote spheroid cell migration and for proper cluster formation and spheroid cell viability in a subset of ovarian cancer cell lines. Ectopic Snail expression in Hey cells enhanced spheroid cell adhesion, invasion and migration potential, which ultimately contributed to increased tumour-forming and metastatic potential. Snail expression has been observed in primary ovarian carcinomas, ovarian cancer effusions and metastases, and associated with shorter overall and progression-free survival [27]. Notably, Snail expression has shown to be significantly higher in later stage ovarian tumours (III and IV) compared to early-stage ovarian tumours [14, 28]. This data clearly implicates Snail in EOC disease progression, and we now provide essential insight into its functional role using an experimentally-tractable and relevant model of EOC metastasis.

In epithelial ovarian cancer, the expression of EMT related transcriptional repressors along with the loss of E-cadherin has been shown to be related to metastatic and recurrent tumour progression. Snail expression in ovarian cancer portends poor patient prognosis [29] and platinum resistance [30]. Surprisingly, there are a limited number of studies examining Snail function in the progression of EOC. Lu et al. [31] showed that *SNAI1* overexpression increased cell proliferation, migration, invasion in adherent cell culture, while *SNAI1* knockdown reduced these effects. *SNAI1*-overexpressing SKOV3 cells exhibited higher rates of tumour growth and larger tumour volumes relative to *SNAI* expressing control cells. These effects were not observed by altered *SNAI2* (Slug) expression, consistent with our previous findings that Slug is not involved in EMT during

EOC spheroid formation [10]. *SNAI1* silencing in ES-2 and SKOV3 cells resulted in decreased MMP-2 and reduces Matrigel invasion in adherent culture [9]. We show that *SNAI1* knockdown in EOC patient ascites cells severely reduces spheroid cell migration, and *SNAI1* knockdown in the Ovcar3 and iOvCa147-E2 ovarian cancer cell lines reduces spheroid cell viability. Conversely, *SNAI1* ectopic expression in Hey cells increases anchorage-independent growth, migration, and invasion in 3D spheroid culture assays. Given the mode of EOC metastasis, it is particularly relevant to study Snail function in the context of spheroids in suspension culture.

The biological significance and clinical relevance of a 3D cell culture system is well-supported in ovarian cancer research, as well as in other cancers. It is important to note that our results where we have altered Snail expression in ovarian cancer cells substantiate the importance of studying spheroids as compared with adherent, proliferating cells. Spheroids more closely mimic the cell-cell, cell-matrix interactions, metabolic gradients, cellular viability and differentiation of malignant cells within a solid tumour than do conventional adherent, monolayer cultures [32, 33]. As such, recent investigations have focused on the invasive properties of ovarian cancer spheroids and their involvement in metastatic dissemination. For example, Iwanicki et al. [34] showed that ovarian cancer multicellular spheroids attach to monolayer mesothelial cells and promote cell clearance by integrin- and force-dependent processes. Expression profiles comparing ovarian cancer spheroids that were competent or incompetent to clear mesothelial monolayers yielded distinct differences in mesenchymal and epithelial cell marker expression [35]. In fact, overexpression of *SNAI1, ZEB1, or TWIST1* in clearance-incompetent cell lines significantly increased mesothelial clearance ability. Thus, ovarian cancer spheroids possessing enhanced mesenchymal characteristics are more efficient at metastasis. Our research substantiates these findings since HeySNAIL spheroids have enhanced adhesion, migration, and invasion potential both in cell culture- and *in vivo*-based assays than control spheroid cells.

We found HeySNAIL spheroids had markedly enhanced adhesion to and invasion through fibronectin-containing matrices. This may have clinical relevance as several isoforms of fibronectin are abundant in the ascites and underlying the mesothelium, which

covers the peritoneum and omentum [5, 36]. In other *in vitro* models of omental metastasis, EOC cells have increased ability to adhere to fibronectin matrices [26]. Indeed, ovarian cancer cell lines express fibronectin-specific integrin isoforms, which are required for efficient EOC cell adhesion [25]. We further investigated spheroid adhesion and invasion using an *ex ovo* chick embryo CAM assay. Snail-expressing spheroids generated larger, well-defined, and more invasive tumour xenografts. Surprisingly, we observed a trend towards greater liver metastasis by invasive HeySNAIL spheroid xenografts, a result that certainly supports the invasion assays performed in cell culture. Of note, a recent study has implicated blood-borne metastasis in EOC, a concept that has been largely underappreciated in ovarian cancer research [37].

Taken together, our data strongly suggest that Snail expression in ovarian cancer spheroids potentiates adhesion and invasion of these multicellular structures, thereby resulting in an enhanced capacity to seed secondary tumour formation. Such results raise the possibility that inhibition of Snail could reduce seeding of new ovarian cancer metastatic lesions following surgical debulking, thereby minimizing the possibility of recurrent metastatic disease. Thus, the findings of our study point out that Snail, and perhaps spheroid-induced EMT in general, could represent an attractive and effective target in EOC therapeutics.

3.5 References

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Table S3.1 Clinical data for ovarian cancer patient-derived samples.

Sample	Age	Histological Subtype	Grade	Stage
EOC82	44	Serous	High	IIIC
EOC138	64	Serous	High	IIIB
EOC148	67	Serous	High	IC
EOC154	66	Serous	High	IIIC
EOC207	65	Serous	High	n.a.
EOC237	54	Serous	High	IIB
EOC238	70	Serous	High	n.a.

n.a.: not available

Chapter 4

4 DISCUSSION

4.1 Summary of findings

Epithelial ovarian cancer is the most lethal gynecologic cancer in the developed world. Due to lack of obvious symptoms and effective screening tests, the diagnosis of ovarian cancer is often made when the disease is at an advanced stage. At this point, disease had spread beyond the primary site, and wide-spread dissemination of cancer cells is found within the peritoneal cavity**,** mainly in the form of spheroids. Despite aggressive surgical and chemotherapeutic interventions, most measures are ineffective on these cell structures in the peritoneum, which are likely the cause of disease recurrence. As such, mortality rates associated with high-grade serous ovarian cancer remain high and have been directly attributed to intra-peritoneal/ intra-abdominal metastases. Therefore, experimental models that allow us to better comprehend the underlying pathobiology of EOC metastasis are of paramount importance to unveil more effective treatment options for patients with late-stage disease. Our studies specifically use a highly-relevant, tractable, non-adherent culture system to examine the molecular underpinnings of multicellular spheroid formation and subsequent reattachment to an adherent substratum.

I specifically focused on the TGFβ signalling pathway and its downstream target gene *SNAI1*/Snail and investigated their roles in spheroid-induced EMT and metastatic potential. Using ascites-derived specimens from high-grade serous ovarian cancer patients, we observed that epithelial ovarian cancer cells induce an endogenous EMT response upon spheroid formation (Chapter 2). I also observed the plasticity of this process, since the cells undergo MET to revert back to an epithelial phenotype upon spheroid reattachment to a substratum. Importantly, I identified that intact TGFβ signalling is required to elicit this EMT response in spheroids since the potent small molecule inhibitor of the TGFβ type I receptor, SB-431542, blocks spheroid-induced EMT. Additionally, SB-431542 treatment upon spheroid re-attachment forces a more potent epithelial phenotype of dispersing cells, thereby decreasing spheroid cell motility and migration. In fact, I found spheroid formation

to be significantly compromised by exposure to SB-431542, and this correlated with a reduction in cell viability, particularly in combination with carboplatin treatment.

Since Snail was observed to be the most robustly-upregulated EMT marker naturally induced upon EOC spheroid formation, and severely reduced by SB-431542, my next objective was to investigate the role of Snail in directly mediating the malignant potential of EOC spheroids (Chapter 3). The functional role of Snail in EOC metastasis, particularly in spheroids, is largely unknown. Via *SNAI1* knockdown experiments, I show that Snail is required to promote spheroid cell migration and for proper cluster formation and spheroid cell viability in a subset of ovarian cancer cell lines. We also retrovirally-transduced the Hey ovarian cancer cell line to ectopically express Snail. HeySNAIL cells exhibited a strong EMT phenotype in adherent culture, a result which correlated with significantly enhanced spheroid cell migration, adhesion and invasion to fibronectin and collagen matrices. Further to this, using an *ex ovo* chick embryo chorioallantoic membrane assay, Snail-expressing EOC spheroids possessed significantly enhanced tumour-forming potential, invasion, and liver metastasis. This data clearly implicates Snail, a direct target gene of TGFβ signalling and a master regulator of EMT, in EOC disease progression.

4.2 The unique and inherent epithelial/mesenchymal plasticity of EOC cells uncovered

Epithelial-to-mesenchymal transition is a universally accepted phenomenon that occurs in the malignant progression of most if not all human carcinomas [1]. Dynamics of EMT during EOC metastasis, particularly with its reversal, or MET, upon secondary tumour formation, has been discussed in the literature [2, 3]. Although EMT has been implicated in disease progression, the majority of investigations have studied the expression of EMT markers and their related transcription factors in stable cell lines, which are induced to undergo EMT either by forced expression of selected transcription factors or after prolonged exposure to cytokines such as TGFβ [1, 4, 5]. So far, very limited analysis has been performed in fresh cultures directly from patient specimens. Moreover, most studies have focused on studying the phenomenon of EMT in 2D cell culture systems, which do not properly recapitulate the *in vivo* tumour microenvironment. In contrast, my

studies focused on investigating the expression of EMT-related markers in adherent and spheroid cell cultures derived from malignant ascites cells of patients afflicted with high-grade serous EOC. We employed a biologically-relevant, tractable *in vitro* model system that takes into account the unique mode of ovarian cancer metastasis, whereby multicellular spheroids represent an important conduit through which cells are able to survive until they reach a mesothelial surface where re-implantation and invasion are possible. Using this model, my studies revealed that ovarian cancer cells within multicellular aggregates or spheroids naturally undergo an EMT response, acquiring more mesenchymal characteristics, which likely prepares these structures to reattach onto substratum.

CDH1/E-cadherin expression was markedly reduced in spheroids compared with adherent cells, in concert with a robust up-regulation of several transcriptional repressors, particularly *SNAI1*/Snail, as well as *TWIST1/2*, and *ZEB2*. In fact, we observed EOC spheroids to naturally undergo the reverse, MET, during reattachment and dispersion onto substratum. Reversed expression changes of the transcriptional repressors were rapid and sustained, although the levels of E-cadherin expression upon spheroid re-attachment did not increase to initial levels completely. In fact, E-cadherin expression of ovarian carcinoma cells floating in ascites and at metastatic sites has been found to be lower than in the primary ovarian tumour [6]. This delay towards a complete MET may perhaps facilitate spheroid cell motility and invasion in the very initial phases of spheroid re-attachment to substratum. In fact, Kumar *et al.* demonstrated that 3D spheroid cultures formed from lung cancer A549 cells show higher migration rates than 2D monolayer cultures in an *in vitro* Transwell assay [7].

It was interesting to us that an EMT response would be induced in EOC spheroids since these cells would not necessarily require a mesenchymal phenotype in these fairly static structures. However, one could envision that an EMT phenotype of spheroids primes the resident cells for invasive capabilities upon their subsequent reattachment. Indeed, evidence from the Brugge lab has demonstrated, in a subset of ovarian cancer cell lines, that ovarian cancer spheroids use myosin-generated force in order to displace the mesothelial layer of cells and gain access to the underlying ECM and promote invasion [8]. In a subsequent study, they correlated such findings with the expression of mesenchymal

and epithelial markers as they demonstrated tumour cells expressing genes associated with a mesenchymal program display more effective mesothelial clearance [9]. Furthermore, endogenous induction of EMT during cell aggregation into spheroid structures may actually be a mechanism EOC cells employ in order to avoid cell death in suspension. In fact, it has been reported in the literature that the sensitivity of cells to undergo anoikis can be placed along a continuum with epithelial cells being the most susceptible and fibroblasts, or mesenchymal-like cells, being the least [10]. Indeed, when we blocked the endogenous induction of EMT in our system (via TGFβ signalling inhibition), EOC cells failed to form compact spheroids. These less cohesive aggregates further correlated with a reduction in cell viability. Others have investigated the expression of EMT-related markers in adherent and spheroid cell cultures derived from malignant pleural effusions of patients affected by lung adenocarcinoma. In comparing EMT marker expression levels in adherent versus spheroid cells, they observed an extremely variable pattern of mesenchymal gene expression from sample to sample; however, the one sample that did present typical features of EMT (upregulation of Snail, Slug, and Twist) gave rise to the most compact, large, and structured spheres [11]. This suggests that EMT may be an important property for proper cell aggregation and spheroid formation.

We observed a very consistent and robust upregulation of *SNAI1*/Snail upon spheroid formation in every single ascites-derived EOC specimen tested, however this was not the case for the closely related transcription factor *SNAI2*/Slug. Although Snail and Slug transcription factors share a high degree of homology, and both have been alluded to as ”functionally equivalent” in terms of EMT inducers and E-cadherin repressors [12-14], significant differences have been observed in their *in vitro* binding affinities to the E-box elements in the promoters of their target genes. When compared, Snail binds to these sequences with a higher affinity than Slug and is a more potent inhibitor of *CDH1* and other target genes (*PTEN*/PTEN, *MUC1*/Mucin1, *CLDN*/Claudin, and *OCLN*/Occludin) [15]. The biological difference between both factors is also highlighted by the distinct effect of genetic deletion of *SNAI1* or *SNAI2* genes in embryonic development: *SNAI1* knockout mice are embryonic lethal [16] while *SNAI2* knockout mice are viable [17]. Furthermore, functional knockdown studies revealed a specific role for Snail and Slug in the tumourigenic and metastatic behavior of squamous carcinoma cells [18]. A distinct role

for both factors has also become apparent in other carcinomas, including EOC [19-24], which supports our findings that *SNAI2*/Slug expression is not consistently altered during spheroid formation and reattachment.

Several groups have also reported that other mesenchymal markers such as vimentin and N-cadherin (typically replacing E-cadherin) are commonly induced during "classical EMT" in carcinomas [25-29]. We did not observe such occurrences in our model system. In fact, the extent of cellular and molecular changes that occur along the pathway towards EMT depends on both the cell type and the number of acquired oncogenic mutations. Some epithelial cells undergo only a limited amount of change towards EMT. Even during developmental EMT, the direct and full conversion from an epithelial to a mesenchymal state is not observed in all species [30]. Nevertheless, even small alterations in migration and cellular plasticity can impact invasion and metastasis significantly [31]. In certain model systems, epithelial cells can undergo a complete loss of expression of all epithelial molecular markers accompanied by acquisition of a completely fibroblastoid phenotype. This is specifically true in the mouse skin model of chemically-induced carcinoma, where the spindle phenotype is driven by TGFβ, but dependent on synergy with activation of the oncogenic H-ras signalling pathway [32, 33]. We did not observe such an overt EMT in our studies. Taken together, the ability of EOC cells to transition into mesenchymal cells and back, either partially or fully, illustrates an inherent plasticity of ovarian carcinoma cells. It may be that this dynamic plasticity is a particularly unique and advantageous characteristic of metastatic ovarian cancer, not unlike its almost exclusive mode of intraperitoneal spread which is unique when compared among other carcinomas.

4.3 Endogenous TGFβ signalling regulates spheroid-induced EMT

To assess the mechanism by which spheroid-induced EMT occurs, I focused on investigating the role of the TGFβ signalling pathway, not only because it's the prototypical inducer of EMT [34], but also because we have previously observed increased TGFβ1 expression in EOC ascites-derived spheroids compared to adherent cells [35]. Indeed, inhibiting TGFβ signalling during EOC spheroid formation using a potent TβRI small molecule inhibitor, SB-431542, efficiently blocked the EMT phenotype in spheroids,

rendering these structures much less cohesive and more vulnerable to cell death, specifically in combination with carboplatin treatment. Moreover, treating EOC spheroids with SB-431542 at the time of reattachment not only dissolved spheroid cell cores, but also enhanced the epithelial phenotype of dispersing cells and severely affected their motility and migration capacity. It has been suggested in the literature that cells with a higher propensity for cell aggregation and compact spheroid formation also have an enhanced invasive capacity within 3D matrices [36]. TGFβ treatment of ovarian cancer cell lines and breast MCF10A1 cells (and its malignant derivatives) has shown to improve cell aggregation and invasion, respectively, in a 3D context [36, 37]. In the 3D breast cancer model system, expression of Smad3/4 and MMP2/MMP9 was found to be important in TGFβ-induced spheroid cell invasion [38]. Furthermore, in the IGROV1 ovarian cancer cell line, treatment of cells with TGFβ1 was shown to increase size and number of spheroids, as well as induce expression of stem-like markers [39].

Results of my studies are the first to show that active maintenance of endogenous TGFβ signalling is required to promote the EMT phenotype in EOC spheroids and their potential to re-attach and spread. My data also suggests that TGFβ signalling plays a critical role in maintaining cell viability and achieving chemo-resistance in ascites-derived EOC spheroids. As such, TGFβ signalling may be essential to promote late-stage ovarian cancer progression, thus serving as a potential therapeutic target to reduce intraperitoneal spread and disease burden upon recurrence.

In fact, small-molecule inhibitors targeted against TGFβ receptors have been effective in different types of cancer in preclinical trials [31]. For example, efficacy of TGFβ receptor kinase inhibitors, such as A-77, Ki26894 and LY2109761 was demonstrated in preclinical studies of gastric cancer, breast cancer and pancreatic cancer [40-42]. A study performed by Sheen *et al*. showed that EW-7203 (a TβRI inhibitor) had suppressive effects on mammary cancer metastasis to the lung using a Balb/c xenograft model system. EW-7203 was shown to efficiently inhibit TGFβ-induced Smad signalling and EMT *in vitro* and *in vivo* [43]. Ge and colleagues published similar results for a different TβRI kinase inhibitor, SD-093 [44]. Other examples of small molecules that block the catalytic activity of TβRI (SB-431542, SD-208 and SM16) have been reported to be

potent antitumour or antimetastatic agents [45-47]. Therefore, in future studies, it would be intriguing to test the numerous other TGFβ signalling inhibitors available [48], and apply them in an *in vivo* intraperitoneal ovarian cancer metastasis model.

4.4 The induced expression of *SNAI1*/Snail in EOC spheroids acts to facilitate disease progression

Because the underlying pathobiology of EOC spheroid cells was dramatically altered by inhibiting the TGFβ signalling pathway, I was curious to investigate which downstream target genes are necessary for the metastatic characteristics of ovarian cancer spheroids that we've observed. Since Snail was consistently and most robustly induced upon EOC spheroid cell formation, and severely reduced by SB-431542, I sought to investigate its role in EOC progression. Expression of Snail was knocked down using si*SNAI1*, in both ovarian cancer cell lines and EOC ascites-derived cells, using our experimentally tractable *in vitro* model system. Snail was shown to be required for proper cluster formation and spheroid cell viability in a subset of ovarian cancer cell lines. Although knockdown of *SNAI1* led to a modest reduction in spheroid formation and cell viability in ascites-derived cells, we observed a severe reduction in the ability of spheroids to migrate due to loss of Snail expression. Furthermore, *SNAI1* ectopic expression in Hey cells (we denoted as 'HeySNAIL' cells) showed no differences in cell viability when compared to control Hey cells, but displayed enhanced adhesion, migration, and invasion potential across different ECM matrices in our 3D spheroid culture assays. This was in contrast to the lack of motility differences seen in 2D scratch wound assays, again emphasizing the importance of using 3D cell culture systems that better mimic physiological features found in solid tumours [49]. Thus, Snail is crucial for the full migratory and invasive properties of these multicellular aggregates.

To assess whether ectopic Snail expression in Hey spheroid cells would enhance their ability to attach and form secondary tumours, akin to ovarian cancer *trans*-coelomic metastasis mechanisms, I used an *ex ovo* chick embryo chorioallantoic membrane (CAM) assay. Using the *ex ovo* chick CAM as a xenograft model facilitates the rapid and direct visualization of tumour growth over a duration of only 7 days [50, 51]. Using this model,

Snail-expressing spheroids generated larger, well-defined, and more invasive tumour xenografts than control Hey spheroids. We also observed a trend towards greater liver metastasis by invasive HeySNAIL spheroid xenografts, a result that certainly supports the results from our invasion assays performed in cell culture. Taken together, our results strongly suggest that Snail expression in ovarian cancer spheroids potentiates adhesion and invasion of these multicellular structures, thereby resulting in an enhanced capacity to seed secondary tumour formation.

To complement these studies in the future, we wish to perform similar chick CAM assays in high expressing Snail cell lines where *SNAI1* would be silenced. We have used an RNAi-mediated knockdown approach to target *SNAI1* in EOC cells and cell lines in our *in vitro* cell culture assays. This method is commonly used because it's rapid and inexpensive, however knockdown may be incomplete and its inhibition is temporary [52]. Recently, the CRISPR/Cas9 (clustered Regularly Interspaced Short Palindromic Repeats/ CRISPR-associated 9) system has been developed to enable the precise editing of specific genomic loci and facilitate the elucidation of target gene function [53]. Briefly, CRISPR "spacer" sequences are transcribed into short RNA sequences capable of guiding the system to matching sequences of DNA. When the target DNA is found, Cas9, one of the enzymes produced by the CRISPR system, binds to the DNA and cuts it. In the absence of a repair template, small insertion or deletion mutations known as indels are formed, leading to potential frameshift and truncating mutations, thus shutting off targeted gene expression [54]. Using modified versions of Cas9 and other CRISPR enzymes, this technique has shown to be a very efficient tool to permanently modify gene expression [55]. In fact, a recent study has used this system to successfully target *SNAI1* in serous RMG-1 ovarian cancer cells [56]. It would be interesting to apply this into a mouse model of intraperitoneal metastasis, and assess ascites generation. Ascites would be harvested from these animals, and effect of *SNAI1* knockout on spheroid formation potential assessed. The cavities of these mice could further be examined to directly assess the ability of spheroids to colonize and invade surfaces. As such, effects of silencing *SNAI1* on spheroid formation, tumour progression, and metastasis could be directly observed in an *in vivo* setting, to further complement our discoveries in a field where the functional role of Snail on the underlying pathobiology of EOC spheroids has not been explored.

To summarize, targeting Snail alone did not fully recapitulate the SB-431542 effects observed on EOC spheroids, indicating that Snail is not the sole mediator of the intrinsic TGFβ-induced EMT we observed in EOC spheroids. In other words, targeting Snail alone *in vitro* may not be entirely successful in blocking all of the EMT properties that we observed in EOC spheroids, and this is likely due to the presence of multiple factors that function together to reinforce the phenotype observed in EOC spheroids. Regardless, my studies demonstrate that Snail is important for the adhesive, migratory and invasive properties of EOC spheroids, and their resultant capacity to seed secondary tumours. Therefore, targeting Snail, or the upstream TGFβ signalling pathway that regulates EMT and Snail in spheroids, in late stage ovarian cancer patients is an attractive therapy that could minimize the possibility of recurrent metastatic disease.

4.5 Conclusion

Epithelial ovarian cancer has one of the poorest prognoses among human neoplasms. The goal of this thesis was to contribute to our knowledge of EOC metastasis, particularly focusing on multicellular spheroids as major contributors to formation of secondary metastatic lesions.

It has been proposed that cancer cells hijack EMT transcriptional programs to dissociate from the primary tumour, intravasate and extravasate through the vasculature, and invade distant sites [1, 57-59]. Ovarian cancer cells do not face these barriers in metastasizing to the peritoneum. Instead, they are displaced from primary tumours directly into the peritoneal cavity, in which single cells aggregate into clusters and acquire survival mechanisms that allow them to transit to other peritoneal tissues and colonize new sites. We have discovered that EMT is naturally induced upon EOC cell aggregation into spheroid structures, a phenomenon that is critical for their survival and malignant behavior. We further found that endogenous signalling by the TGFβ signalling pathway regulates this spheroid-induced EMT, and its downstream target Snail is crucial for the malignant properties that mediate spheroid cell invasion, spread, and secondary tumour formation. Understanding the unique characteristics afforded to non-adherent ovarian cancer cells is critical for the identification of more effective treatment regimens particularly for late-stage

recurrent disease. Overall, this body of work has contributed to the field of ovarian cancer metastasis providing rationale for investigating the TGFβ/EMT/Snail pathway further in preclinical models.

4.6 References

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Curriculum Vitae

Samah Rafehi

Education

2011 -Present Doctor of Philosophy (PhD) in Anatomy and Cell Biology
- The University of Western Ontario, London, Ontario
- Department of Anatomy and Cell Biology.

2010 - 2011 Doctor of Philosophy in Pathology
- The University of Western Ontario, London, Ontario
- Department of Pathology
- Transfer to department of Anatomy and Cell Biology.

2008 - 2010 Masters in Science (MSc)
- The University of Western Ontario, London, Ontario
- Clinical Anatomy, Department of Anatomy and Cell Biology
- Course-based Masters with research component

2006 - 2008 Bachelor of Medical Sciences (BMSc)
- The University of Western Ontario, London, Ontario
- Honors specialization in Medical Sciences with Distinction

2003 – 2006 Bachelor of Biology (BSc)
- Notre Dame University, Al-Koura, Lebanon
- Honors with High distinction (Summa Cum Laude)
- credits transferred to BMSc

Teaching Experience

2011 - 2015 Teaching Assistant (TA) – Medical Science Laboratory (4930F/G)
- TA for 4th year undergraduate Medical Science Students

2011 - 2013 Teaching Assistant (TA) – OT Gross Anatomy Laboratory (course 9524)
- Anatomy TA for Occupational Therapy Students
- Direct student learning in the lab with pre-dissected cadaveric specimens

2009 - 2010 Teaching Assistant (TA) – Medicine Gross Anatomy Lab
- Anatomy TA for 1st and 2nd year Schulich Medicine Students
- Guide student learning in the lab by helping with dissections and answering student questions; also responsible for marking student assessments

2008 - 2009 Teaching Assistant (TA) – Dentistry Gross Anatomy Lab

- Anatomy TA for 1st year Schulich Dentistry Students
- Guide student learning in the lab by helping with dissections and answering student questions; also responsible for proctoring lab exams

Presentations / Abstracts

06/2015 Oncology Research and Education Day
- The Lamplighter Inn, London, Ontario
- Poster presentation "TGFβ Signalling Regulation of the EMT Phenotype in Ascites-Derived Epithelial Ovarian Cancer Spheroids"

05/2015 Paul Harding Research Day
- London Health Sciences Centre, Victoria Hospital, London, Ontario
- Podium presentation "TGFβ Signalling Regulation of the EMT Phenotype in Ascites-Derived Epithelial Ovarian Cancer Spheroids"

03/2015 London Health Research Day
- London Convention Center, London, Ontario
- Poster presentation "TGFβ Signalling Regulation of the EMT Phenotype in Ascites-Derived Epithelial Ovarian Cancer Spheroids"

02/2015 Anatomy and Cell Biology Seminar Series
- The University of Western Ontario, London, Ontario
- Podium presentation "Exploring the regulation and function of epithelial-mesenchymal plasticity in ovarian cancer spheroids"

12/2014 Cancer Research Lab Presentation (CRLP)
- London Regional Cancer Program, Victoria Hospital, London, Ontario
- Podium presentation "Investigating the requirement of the transcriptional repressor, SNAIL, in EOC spheroids"

06/2014 Oncology Research and Education Day
- The Lamplighter Inn, London, Ontario
- Poster presentation "TGFβ Signalling Regulation of the EMT Phenotype in Ascites-Derived Epithelial Ovarian Cancer Spheroids"

05/2014 Canadian Conference on Ovarian Cancer Research (CCOCR)
- Victoria, BC

- Poster presentation "TGFβ Signalling Regulation of the EMT Phenotype in Ascites-Derived Epithelial Ovarian Cancer Spheroids"
- *Presented by supervisor*

05/2014 Paul Harding Research Day
- London Health Sciences Centre, Victoria Hospital, London, Ontario
- Podium presentation "TGFβ Signalling Regulation of the EMT Phenotype in Ascites-Derived Epithelial Ovarian Cancer Spheroids"

03/2014 London Health Research Day
- London Convention Center, London, Ontario
- Poster presentation "TGFβ Signalling Regulation of the EMT Phenotype in Ascites-Derived Epithelial Ovarian Cancer Spheroids"

02/2014 Cancer Research Lab Presentation (CRLP)
- London Regional Cancer Program, Victoria Hospital, London, Ontario
- Podium presentation "TGFβ/BMP Signalling Regulation of the EMT Phentoype in Ascites-Derived Epithelial Ovarian Cancer Spheroids"

06/2013 Oncology Research and Education Day
- The Lamplighter Inn, London, Ontario
- Poster presentation "TGFβ Signalling Regulation of the EMT Phenotype in Ascites-Derived Epithelial Ovarian Cancer Spheroids"

05/2013 Paul Harding Research Day
- London Health Sciences Centre, Victoria Hospital, London, Ontario
- Poster presentation "TGFβ Signalling Regulation of the EMT Phenotype in Ascites-Derived Epithelial Ovarian Cancer Spheroids"

03/2013 London Health Research Day
- London Convention Center, London, Ontario
- Poster presentation "TGFβ Signalling Regulation of the EMT Phenotype in Ascites-Derived Epithelial Ovarian Cancer Spheroids"

10/2012 Anatomy and Cell Biology Research Day
- The University of Western Ontario, London, Ontario

- Poster presentation "TGFβ/BMP Signalling Regulation of the EMT Phenotype in Ascites-Derived Epithelial Ovarian Cancer Spheroids"

06/2012 Oncology Research and Education Day
- The Lamplighter Inn, London, Ontario
- Poster presentation "TGFβ/BMP Signalling Regulation of the EMT Phenotype in Ascites-Derived Epithelial Ovarian Cancer Spheroids"

05/2012 Canadian Conference on Ovarian Cancer Research (CCOCR)
- Chateau Laurier, QC, Quebec
- Poster presentation "TGFβ/BMP Signalling Regulation of the EMT Phenotype in Ascites-Derived Epithelial Ovarian Cancer Spheroids"

05/2012 Paul Harding Research Day
- London Health Sciences Centre, Victoria Hospital, London, Ontario
- Poster presentation "TGFβ/BMP Signalling Regulation of the EMT Phenotype in Ascites-Derived Epithelial Ovarian Cancer Spheroids"

03/2012 London Health Research Day
- London Convention Center, London, Ontario
- Podium presentation "Elucidating the Effects of TGFβ/BMP Signalling on Epithelial Mesenchymal Transition in Epithelial Ovarian Cancer"

03/2012 Cancer Research Lab Presentation (CRLP)
- London Regional Cancer Program, Victoria Hospital, London, Ontario
- Podium presentation "TGFβ/BMP Signalling Regulation of the EMT Phentoype in Ascites-Derived Epithelial Ovarian Cancer Spheroids"

2011 Canadian Orthopedic Association (COA)
- St. John's Convention Center, St. John's, Newfoundland
- Podium presentation "An Anatomic Study of Coronoid Cartilage Thickness with Special Reference to Fractures"

2010 Mayo Clinic Elbow Club Meeting
- Mayo Clinic, Rochester, MN
- Podium presentation "Coronoid Fractures: Fleck Off?"
- *Presented by supervisor*

2010 Fifth Annual Imaging Discovery Conference
- London Convention Center, London, Ontario
- Poster presentation "An Anatomic Study of Coronoid Cartilage Thickness with Special Reference to Fractures"

2010 Experimental Biology Conference
- Anaheim Convention Center, Anaheim, California
- Poster presentation "An Anatomic Study of Coronoid Cartilage Thickness with Special Reference to Fractures"
- Received Travel Award

2010 Margaret Moffat Research Day
- The University of Western Ontario, London, Ontario
- Poster presentation "An Anatomic Study of Coronoid Cartilage Thickness with Special Reference to Fractures"
- Award Winner

2009 The 16th Annual Murray Barr Research Day
- University of Western Ontario, London, Ontario
- Podium presentation "An Anatomic Study of Coronoid Cartilage Thickness with Special Reference to Fractures"

2009 Guest Lecturer
- University of Western Ontario, London, Ontario
- Anatomy and Cell Biology course 3319/2225

Publications

Rafehi Samah, Ramos-Valdes Yudith, Bertrand Monique, McGee Jacob, Prefontaine Michel, Sugimoto Akira, DiMattia Gabriel, Shepherd Trevor. TGFβ signalling regulates EMT in ovarian cancer ascites-derived spheroids. ***Endocrine-Related Cancer****.2015*

Rafehi Samah, Lalone Emily, Johnson Marjorie, King Graham, Athwal George. An anatomic study of coronoid cartilage thickness with special reference to fractures. ***J Shoulder Elbow Surg***. 2011

Academic Awards/Scholarships

2015 13th Annual Paul Harding Research Day- Second Prize Award
- Value of award given: $100

2014 -2015 Obstetrics and Gynecology Graduate Scholarship (OGGS)
- Value of award given: $16,500

2013 -2014 CIHR student training program in Cancer Research Scholarship

- Value of award given: $19,100

2012-2013 Queen Elizabeth II Graduate Scholarship in Science and Technology
- Awarded to graduate students based on academic performance. Value of award given: $15,000/year

2008 - 2013 Western Graduate Research Scholarship (WGRS)
- Awarded to graduate students entering graduate studies with an 80%+ average and who maintain an 80%+ average

2012 Ovarian Cancer Canada (OCC) Teal Heart Scholarship
- Awarded to Canadian graduate students involved in ovarian cancer research. Value of award given: $3,000

2011-2012 Ontario Graduate Scholarship (OGS)
- Awarded to Ontario graduate students at the master's or doctoral levels who have displayed academic excellence. Value of award given: $15,000/year

2010-2011 Ontario Graduate Scholarship in Science and Technology (OGSST)
- Awarded to Ontario graduate students at the master's or doctoral levels who have displayed academic excellence. Value of award given: $10,000

2010 The Gabriel G. Altman Research Award, University of Western Ontario
- Awarded for an outstanding Poster Presentation at the Margaret Moffat Research Day

2010 American Association of Anatomists (AAA) Travel Award, AAA

2008 – 2010 Schulich Graduate Research Scholarship (SGRS)
- Awarded by the Schulich School of Medicine and Dentistry to graduate students who are admitted with and maintain an 80%+ average

2009 Ontario Graduate Scholarship (OGS)
- Awarded to Ontario graduate students at the master's or doctoral levels who have displayed academic excellence. Value of award given: $15,000/year.

2004 - 2006 Notre Dame Summa Cum Laude Scholarship, Notre Dame University
- Covered 75% of tuition – awarded to undergraduate students with exceptional academic achievement (minimum GPA of 3.8)

2003 Notre Dame Scholarship of Excellence - Entrance Scholarship

- Covered 25% of tuition - awarded to high school graduates entering with an average of 85% or higher

Extracurricular Activities

2008- 2014

- Let's Talk Science volunteer
- St. Leonard's Justice Committee member

2006- 2008

- Canadian Blood Services volunteer
- Nurse's Aide, Parkwood Hospital