Investigation of $\beta 5$ integrin function in epithelial ovarian cancer cell adhesion and metastatic properties of spheroids

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Abstract

Epithelial ovarian cancer (EOC) is the most lethal gynecological malignancy in the developed world. EOC metastasis is unique since malignant cells detach directly from the primary tumor site into the abdominal fluid and form multicellular aggregates, called spheroids, that possess enhanced survival mechanisms while in suspension. As such, altered cell adhesion properties are paramount to EOC metastasis with cell detachment from the primary tumor, dissemination as spheroids, and reattachment to peritoneal surfaces for secondary tumor formation, that play a crucial role in cell-cell and cell-extracellular matrix interactions, having implications in multiple steps of cancer progression. We previously showed that the CRISPR-ablation of LKB1 or its downstream effector NUAK1 resulted in spheroid disaggregation \textit{in vitro} and is required for efficient EOC metastasis in mouse tumor cell xenografts. Global gene expression analysis demonstrated a coordinated reduction in $\beta_5$-integrin (encoded by \textit{ITG$\beta$5} gene). Integrins are a family of cell-adhesion receptors required to mediate cellular interactions with the extracellular matrix (ECM) and promote tumorigenesis in various malignancies; however, the role of $\beta_5$-integrin in EOC is unknown. Using publicly-available datasets and western blot analysis, we identified relatively high $\beta_5$ integrin expression in established and patient ascites-derived EOC cell lines. siRNA-mediated knockdown of \textit{ITG$\beta$5} reduced EOC cell adhesion, impacted adherent cell and spheroid viability. We identified that $\beta_5$ integrin is required for efficient spheroid reattachment and subsequent cell spreading. When evaluating the interaction of $\beta_5$ integrin with ECM ligands, results indicate that $\beta_5$ integrin and its association with vitronectin may play a role in spheroid reattachment.

\textbf{Keywords}: $\beta_5$ integrin, adhesion, reattachment, spheroid, ovarian cancer, metastasis
Summary for lay Audience

Epithelial ovarian cancer is one of the most lethal gynaecological cancers in the developed world and most individuals are diagnosed at advanced stages. When ovarian cancer cells spread, a process called metastasis, the cancer cells try to survive starvation-like conditions by forming cell clusters called spheroids. During this time, ovarian cancer cells change their cell adhesion properties to allow them to survive and form more tumours. Our laboratory discovered that a molecule called “Liver Kinase B1” (LKB1) and its downstream target, NUAK1, enables ovarian cancer cells to survive these stressful conditions using different strategies to generate energy. The loss of these two molecules leads to a decrease in a cell adhesion molecule called β5 integrin. My work focuses on taking a closer look at the change in cell adhesion properties in ovarian cancer because they are a critical aspect of metastasis. Currently, I am using different functional assessments to determine the role of β5 integrin in cell adhesion and spheroid formation. Understanding how ovarian cancer cells alter their cell adhesion to allow for metastasis may reveal unique vulnerabilities that could improve treatment outcomes for ovarian cancer patients.
Co-Authorship Statement

iOvCa series, ascites-derived cell lines, were established by Dr. Gabriel DiMattia.

Compiling integrin mRNA expression data in ovarian cancer tumors and cell lines from the Cancer Genome Atlas (TCGA) was completed by Dr. Trevor Shepherd.

Gathering β5 integrin mRNA expression data among High-grade serous ovarian cancer and ovarian clear cell carcinoma cell lines from Cancer Cell Line Encyclopedia (CCLE) dataset was completed by Bartlomiej Kolendowski.
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Chapter 1

1. Introduction

1.1. Epithelial ovarian cancer

Epithelial ovarian cancer (EOC) is the most lethal gynecological malignancy in the developed world[1]. Most women are diagnosed with advanced-stage disease with a 5-year survival rate of less than 29%, since 80% of these cases present initially with metastasis[1], [2]. The delay in diagnosis can be attributed to the wide range of non-specific symptoms like abdominal fullness, vaginal bleeding and urinary symptoms thereby leading to a more advanced-stage before clinical assessment[3]. Factors for increased risk include early age of menarche, and benign gynecological conditions such as endometriosis, polycystic ovary and pelvic inflammatory disease, whereas oral contraceptive use and tubal ligation can decrease risk for EOC[1]. The current treatment plan for patients with EOC in which tumors have spread beyond the ovaries is maximal surgical cytoreduction with adjuvant chemotherapy of combined carboplatin and paclitaxel[1]. However, 75% of patients will have disease reoccurrence, oftentimes acquiring chemotherapy resistance, ultimately leading to a dire prognosis[1], [3].

EOC encompasses a heterogenous group of malignant tumors that differ in prognosis, molecular pathology, and etiology. According to the World Health Organization (WHO), EOC can be classified into seven histological subtypes: high-grade and low-grade serous, mucinous, endometrioid, clear cell, Brenner, seromucinous and undifferentiated carcinomas[4]. These histological subtypes can be organized into two major EOC groups where Type I consists of lower grade, slow-proliferating carcinomas within serous, endometrioid, mucinous and clear cell
histological subtypes that likely arise from benign ovarian lesions[4], [5]. Whereas Type II tumors are typified as being more aggressive disease derived from secretory fallopian tube epithelium, and present histologically as high-grade serous, mixed epithelial or undifferentiated carcinomas[5], [6]. High-grade serous ovarian cancer (HGSOC) represents 75% of all cases and is characterized by the near universal presence of TP53 tumor suppressor gene mutations, commonly as observed as missense gain-of-function alterations, although deletions and nonsense loss-of-function mutations have been identified, too[7], [8]. This genetic alteration arises within an early tumor precursor cell of the distal fallopian tube, called serous tubal intraepithelial carcinoma (STIC) lesion; protein-stabilizing TP53 missense mutations promote secretory epithelial cell survival and cell-cell aggregation under anchorage independent growth conditions[8]. HGSOC is associated with lower prevalent but recurrent somatic mutations in NFI, BRCA1, BRCA2, RB1 and CDK12 totalling 5-8% of tumors[1], [8].

Furthermore, advanced EOC is characterized by ascites fluid accumulation within the peritoneal cavity[5]. The impairment of lymphatic drainage and increased secretion of vascular endothelial growth factor (VEGF) leads to enhanced vascular permeability[5]. The unique microenvironment within malignant EOC ascites consists of a variety of non-tumor cell types, such as fibroblasts, mesothelial cells, immune cells and endothelial cells, as well as acellular components, such as soluble extracellular matrix (ECM), matrix-degrading enzymes, cytokines and growth factors[9].

1.2. Extracellular Matrix interactions in EOC

The ECM is an integral and dynamic non-cellular component within all tissues and functions to support cells and maintain tissue homeostasis[9]–[11]. Normal ovarian ECM is composed of a highly-ordered arrangement of collagen fibers and proteoglycans, such as decorin and versican, to
provide structural integrity, and maintain interstitial pressure and hydration to tissue[9]–[11]. However, ECM stiffness is commonly increased in EOC tumors through the activation of stromal fibroblasts and collagen remodeling into thick fibrils in random orientation, which can combine to increase tumorigenesis, cancer invasion and migration[10]–[12]. For example, decorin loss and upregulation of versican, fibronectin, tenascin-C, and tenascin-X are associated with poor prognosis and overall survival in EOC[9], [12]. The binding of various ECM ligands to integrins, which are glycoprotein receptors at the cell surface to promote adhesion, regulate complex signaling events alone or in combination with growth factor receptors[13], [14].

The interactions between the tumor cells and ECM within the tumor microenvironment are crucial since their dysregulation has been implicated in EOC progression[9], [13]. Therefore, integrin-mediated interactions and function within the tumor micro-environment represents a potential unique therapeutic strategy in EOC. In this review, we discuss the contributions of integrin-mediated cell adhesion in the critical steps during intraperitoneal metastatic cascade of EOC pathogenesis, including spheroid formation, epithelial-mesenchymal plasticity, and mesothelial attachment of secondary tumors.

1.3. Integrin signalling

The integrins comprise a superfamily of cell adhesion receptors that recognize ECM and cell-surface ligands[15]. There are 18 α-subunits and 8 β-subunits that assemble to create 24 functionally distinct transmembrane heterodimers. Integrins are grouped according to their ligand-binding specificity: collagen-binding integrins (α1β1, α2β1, α10β1, and α11β1), laminin-binding integrins (α3β1, α6β1, α7β1, and α6β4), leukocyte-integrins (α4β1, α5β1, α4β7, αEβ7, αLβ2, αMβ2, αXβ2, ααβ2,
and αDβ2) and arginine-glycine-aspartate (RGD)-recognizing integrins (α5β1, α8β1, ανβ1, ανβ3, ανβ5, ανβ6, ανβ8, and αυνβ3)[15] (Figure 1.1).

These receptor complexes have no enzymatic activity but instead activate bidirectional signaling pathways[13],[15]. The affinity of integrin receptors for ECM components and other ligands is tightly regulated by inside-out signaling[15]. Integrin receptors maintain α and β subunit cytoplasmic tail association during their inactive stage, and cytoplasmic signals from associated G-protein coupled receptors lead integrin β subunit phosphorylation within its cytoplasmic domain for receptor activation[15],[16]. The integrin binding of cytoskeletal proteins such as talin, vinculin and ERM (ezrin, radixin, and moesin) acts to regulate actin microfilaments of the cytoskeleton[15],[16]. In contrast, outside-in signaling occurs through the clustering of integrin receptors at the plasma membrane where ECM ligation transduces signals intracellularly[15],[17]. Natural extracellular ligands include several components of the ECM such as collagen, laminin, fibronectin and vitronectin[13],[15]. Extracellular ligand binding induces conformational changes in the integrin receptor to allow intracellular tails of the β subunit to engage with intracellular signaling molecules including focal adhesion kinase (FAK), small GTPases Rho and Ras, and adaptors, such as Cas/Crk and paxillin[17]. These activated integrin-ECM interactions lead to the formation of dynamic adhesion structures as small extensions from the plasma membrane called podosomes[15],[16],[18]. After ECM ligation, integrins acting alone or in complex with growth factors present in the local microenvironment can regulate diverse tumor cell functions, such as migration, invasion, adhesion and proliferation through the activation of various signaling pathways[13],[15].
Integrins are a superfamily of cell adhesion receptors that recognize ECM and cell-surface ligand. They consist of 18 α-subunits and 8 β-subunits that assemble to create 24 functionally distinct transmembrane heterodimers. (a) Integrins are grouped according to their ligand-binding specificity: arginine-glycine-aspartate (RGD)-recognizing integrins, laminin-binding integrins, collagen-binding integrins and leukocyte-integrins. (b) Integrins take part in bidirectional signaling once the integrins move from a bent configuration to an active form. During inside-out signaling, G-protein coupled receptors lead to integrin β subunit phosphorylation within the cytoplasmic domain for receptor activation. The integrin binding of cytoskeletal proteins such as talin, vinculin and ERM (ezrin, radixin, and moesin) acts to regulate actin microfilaments of the cytoskeleton. Outside-in signaling occurs through the clustering of integrin receptors at the plasma membrane where ECM ligation transduces signals intracellularly. ECM binding with ligands such as collagen, laminin and fibronectin induces conformational changes in the integrin receptor to allow intracellular tails of the β subunit to engage with intracellular signaling molecules including focal adhesion kinase (FAK), small GTPases Rho and Ras, and adaptors, such as Cas/Crk and paxillin. Integrin acting alone or in complex with growth factors present in the local microenvironment can regulate diverse tumor cell functions, such as migration, invasion, adhesion and proliferation through the activation of various signaling pathways. [created via biorender.com]
1.4. Implications of integrin function in EOC metastasis

The primary site of origin for HGSOC is the secretory epithelial cells of the distal fallopian tube from precursor STIC lesions[7], [19]. After TP53 mutations occur, it is postulated that cells within precursor lesions can be further stimulated by local inflammatory cytokines, growth factors and hormones, such as transforming growth factor-beta (TGF-β) and activin A present in follicular fluid that can stimulate migration of STIC cells to the ovary[20]–[22]. The movement of STIC lesions to the rich microenvironment provided by the ovary is a critical step in the transition of STIC lesions to HGSOC by attaching, invading and establishing a primary tumor[1], [23]–[25]. This model has been supported by studies in which ovariectomies performed in mice harboring precursor lesions results in reduced tumor formation and intraperitoneal metastasis, emphasizing the importance of the ovarian microenvironment for complete malignant progression[23], [26].

Unlike hematogenous routes involving intravasation and extravasation where cancer cells must penetrate multiple barriers, intraperitoneal dissemination is a common form of EOC metastasis, and is also observed less frequently in colorectal, gastric and pancreatic cancers[5], [27], [28]. Despite the more passive nature of intraperitoneal dissemination, it leads to rapid disease progression, frequent relapse, complications like bowel obstruction, and overall poor prognosis[5], [6]. During advanced-stage EOC, metastatic cancer cells impair lymphatic drainage and secrete vascular endothelial growth factor (VEGF) that enhances vascular permeability and ascites fluid accumulation in the peritoneal cavity[5], [29]. Ascites fluid often contains EOC cells, as well as a highly heterogeneous mix of other cellular and acellular components[5], [9]. Direct spread of EOC tumor cells into the peritoneal cavity is due to enhanced anchorage-independent tumor cell survival that may be supported by altered cell-cell and cell-ECM functions of various integrins.
1.4.1. Integrins in EOC spheroids

EOC cells that are bound for metastatic dissemination must first detach from the primary tumor site. Proteolytic activity by membrane type 1 matrix metalloproteinase (MT1-MMP, or MMP14) is required in part for the initial detachment of EOC cells from the primary ovarian tumor by cleaving α3 integrin on cancer cells and contributing to decreased cell adhesion[30]. Detached EOC cells survive in hypoxic and anchorage-independent conditions by forming heterogeneous multicellular structures known as spheroids thereby avoiding anoikis, which is a specific form of apoptosis triggered by the lack of attachment to other cells or ECM[5], [31], [32]. Spheroids further complicate the disease by exhibiting decreased cell proliferation by accumulating in the G0/G1 phase of the cell cycle and becoming resistant to chemotherapeutic agents, such as paclitaxel and cisplatin[33], [34]. It is important to appreciate, however, that not all cells within spheroids may have the same properties. For example, spheroids can move in a coordinated fashion, a process called collective migration. This is driven by outer “leader” cells that direct migration and enable the invasion of basement membrane, then “follower cells” that mediate actomyosin contraction allowing for cellular movement[35], [36].

The first step of spheroid formation consists of cell-cell interactions either directly or through ECM bridges. The abundance of integrins available to cells within these multicellular aggregates may provide a major contribution to spheroid formation and pro-survival signaling[37]. Doberstein et al. demonstrated that the loss of L1 cell adhesion molecule (L1CAM) reduced the capacity for OVCAR8 cells to form spheroids, ultimately leading to cell death[26]. Alternatively, L1CAM overexpression led to increased spheroid formation in OVCAR8 cells as well as multiple immortalized human fallopian tube epithelial cell lines[26]. L1CAM expression promotes the upregulation of fibronectin and integrin subunits α5 and β1, which together promote cell
aggregation into spheroids yet detachment from the primary tumor and tumorigenesis[26]. Fibronectin is abundant within the ascites and plays a critical role in spheroid integrity[38], [39] by interacting with its canonical integrin receptor α5β1[40], [41]. For example, OVCAR5 spheroids co-express of α5β1 integrin and fibronectin on the their surface[41]. The functional inhibition of β1 integrin using an inhibitory antibody results in the disruption of EOC spheroids, whereas β1 integrin clustering and fibronectin activate α5β1 heterodimer assembly to promote spheroid formation[41].

Spheroid compaction into dense aggregates is critical for tumorigenesis and related to their actomyosin contractile capacity mediated by integrins and cadherins[39], [42]. A positive correlation may exist between compact spheroid formation and tumorigenic capacity, as well as enhanced invasive capacity in EOC[42]. Sodek et al. demonstrated that β1 integrin activation using an activating antibody 12G10 and ectopic β1 integrin upregulation enhanced more compact cell aggregation using SKOV3 and OVCAR3 cells, two EOC cell lines that typically form less compact spheroids[42]. Conversely, treating compact HEY cells spheroids with a β1-integrin blocking antibody MAB13 led to spheroid disaggregation[42]. Casey and colleagues also demonstrated that treating OVCAR5 cells with a β1-integrin blocking antibody inhibits spheroid formation, while addition of exogenous fibronectin promoted EOC spheroid formation[41]. Similarly, laminin interactions with α6β1 integrins, and collagen with α2β1-integrins, mediate spheroid formation[41], [43]. In contrast, spheroid formation can be enhanced in the presence of antibodies targeting α2, α4, α6 or α6β3 integrins implicating these integrins may negatively regulate spheroid formation[41].

Kellouche and colleagues identified αv integrin and vitronectin colocalization within multicellular aggregates at intercellular sites suggesting a contribution in cell-cell interactions[44]. They demonstrated through the use of anti-vitronectin, anti-αv integrin, or the cyclic peptide
cRGDFV, blocked initial formation of IGROV1 spheroids[44]. The blockade of αv integrins decreased integrin-linked kinase (ILK) activity resulting in reduced Akt phosphorylation and increased cell cycle inhibitor p27kip1 expression[45]. αv integrin can directly regulate ILK activity since anti-αv integrin inhibits ILK activity whereas ectopic ILK overexpression rescues the inhibitory effect of αv integrin blockade[45]. Anchorage-independent growth of IGROV1 cells leads to a significant decrease in ERK1/2 phosphorylation compared to adherent cells and inhibition of ERK1/2 activation with MEK1/2 inhibitor U0126 in IGROV1 spheroids led to a decrease in the number of viable cells and increase in PARP cleavage and caspase-3 activity[46]. Carduner et al. show that increased anoikis in IGROV1 spheroids due to αv-integrin silencing is associated with decreased ERK1/2 activation. This suggests that αv-integrin promotes spheroid cell survival by inducing ERK-dependent pathways[46]. This association has also been demonstrated in an anoikis-resistant population of human intestinal carcinoma cells due to αvβ3 integrin expression[47].

Cancer stem-like cells (CSC) may play a role in EOC spheroid formation. Exogenous CD90 decreased SKOV3 spheroid formation and promoted apoptosis as seen by increased cleaved poly (ADP-ribose) polymerase expression[48]. Ectopic CD90 expression led to decreased expression of CSC markers CD133 and CD24, and promoted mTOR phosphorylation as well as its downstream target AMPK[48]. However, β3-integrin silencing increased anchorage-independent growth and CD133 marker expression. CD90 is associated with αvβ3 integrin through its regulation of signal transduction in astrocytes and neuronal cells[49]. Taken together, this suggests negative regulatory role of CD90 together with β3-integrin signaling in the context of CSCs and the EOC spheroid phenotype.
Spheroids present in malignant ascites can interact with other cell types to affect their phenotype. For example, an analysis of cell components in spheroids isolated from the ascites of 128 patients with stage III ovarian cancer showed the presence of macrophages in all spheroids[50]. The number of macrophages present with spheroids compared to primary tumors was substantially higher and positively correlated with proliferation in spheroids and negatively with patient prognosis[50]. Robinson-Smith et al. demonstrated spheroid implantation in a mouse model of EOC increased due to inflammation, whereas the loss of peritoneal macrophages reduced metastatic potential, supporting the role of tumor-associated macrophages (TAMs) in EOC[51]. In fact, Yin et al. discovered that EOC spheroids express ICAM1, a ligand that binds leukocyte-associated integrin subunits αM and αx. Blockade of this interaction between EOC cells and TAMs diminished spheroid formation in mouse and human in vitro spheroid coculture models[50] TAMs are a source of epidermal growth factor (EGF), and EGF signaling is critical for EOC cell proliferation to increase VEGF-C and enhance integrin-ICAM1 expression, spheroid formation and migration[50].

1.4.2. Integrins in epithelial-mesenchymal transition and EOC cell migration

Cancer cells destined for dissemination co-express epithelial and mesenchymal markers, commonly referred to as epithelial-mesenchymal transition (EMT), allowing for a cadherin switch[36], [52]. EMT allows ovarian cancer cells to loosen intercellular adhesions between cells contributing to the transition of cells from a primary tumor to shed as single cells or spheroids into the ascites[5], [39]. During this EMT process, cancer cells gain a more invasive properties, survive in hypoxic conditions, and spread through the abdominal cavity by the peritoneal fluid flow[53]. The decrease in cell-cell adhesion and detachment of EOC cells from the primary tumor into the
peritoneal cavity is mediated through the integrin-mediated upregulation of matrix metalloproteinases (MMPs) and activation of EMT[36]. Clustering of collagen-binding integrins α2β1 and α3β1 on EOC cells leads to the induction of MMP9, which is capable of E-cadherin ectodomain cleavage and cell-cell adhesion loosening in an Src kinase-dependent manner[54]. E-cadherin loss leads to transcriptional upregulation of fibronectin receptor α5β1 integrin, which is essential when spheroids initiate adhesion at a secondary site[55]. Decreased E-cadherin is also accompanied by reductions in occludins, claudins, epithelial cell adhesion molecule (EpCAM), α6β4 integrin and cytokines, all of which act to stabilize tight cell-cell contacts via desmosomes[22]. In a reciprocal fashion, there are increases in vimentin, fibronectin, N-cadherin, β1 and β3 integrins and matrix metalloproteinases[22]. Forced downregulation of E-cadherin in EOC cells increases α5 integrin expression through focal adhesion kinase (FAK) and ERK1 activities leading to enhanced cell adhesion to fibronectin[56]. As expected, these EMT-like changes due to E-cadherin loss promote EOC cell invasive properties required for metastasis[53].

TGF-β signaling is widely-recognized as one of the most important pathways required to promote EMT in human cancers. We have demonstrated that TGF-β activity is induced during ascites-derived EOC spheroid formation as indicated by increased mesenchymal marker transcripts, whereas TGF-β signalling inhibition dramatically reduces EMT properties and cell-cell cohesion within spheroids[57]. Bianchi et al. have shown that β integrin subunits associated with αv integrin are upregulated during TGF-β induced EMT in breast carcinoma, and specific integrin β5 downregulation blocked TGF-β induced EMT[58]. Similarly, αvβ8 integrin mediates latent TGF-β activation and resultant EMT in various cancers contributing to cell migration and growth[59].
The ascites microenvironment may play a critical role in promoting a partial shift towards a mesenchymal phenotype in EOC cells[46]. When cultured EOC cells are exposed to ascites, αv integrin localization moves from focal contact structures to intracellular perinuclear vesicles in IGROV1 cells[46]. In a pulse-chase experiment, Carduner et al. showed IGROV1 cells exposed to ascites led to centripetal movement of αv integrin, whereas they remain localized to focal contacts in standard culture medium[46]. Furthermore, the αv integrin cyclic antagonist cRGDFc peptide inhibited multicellular aggregate formation by 40% compared to a non-targeting control peptide[46]. Although these studies suggest that αv-integrin and TGF-β work in concert to control EMT, cell adhesion and migration, a broader role of αv integrin complexes in EOC pathogenesis remains unclear and further investigation is required.

When establishing secondary tumors, spheroids attach to the mesothelium lining through the interactions between spheroids and surface receptors on the mesothelial layer[22], [60]. At this point, spheroid cells induce expression of several integrins that prime the spheroids for attachment to the mesothelium and underlying ECM proteins[22], [41], [61]. For example, interaction between spheroid cells expressing α5β1 integrin receptor and the mesothelium containing fibronectin matrix is essential for spheroid adhesion[41]. Although Cannistra et al. showed that neutralizing antibodies against α5β1 and αvβ3 integrins did not affect the binding of EOC cells to the mesothelium, additional studies by this group and others have shown a partial block in adhesion when inhibitory β1 integrin antibody was administered[41], [62], [63]. Similarly, inhibition of α3, α6 and β1 integrin subunits decrease invasiveness and collagen-binding of spheroids[36]. As the major receptors for ECM proteins, integrins pose as critical regulators of cancer cell adhesion and invasion at a secondary site.
1.4.3. Integrin-mediated mesothelial attachment and migration

The final step of EOC metastasis occurs when spheroids alter adhesion between tumor cells, penetrate mesothelial surfaces, and degrade the ECM within the basement membrane underlying the peritoneum, omentum and abdominal organs[64]. An early step in this process is the integrin-mediated binding of EOC cells to mesothelial cells and exposed ECM[65]. Kaur et al. demonstrated that β3 integrin expression correlates with increased EOC cell adhesion in vitro and adhesion to mesothelium and mouse omentum in vivo[65]. However, they also showed that αvβ3 overexpressing cells inhibited cell Matrigel invasion, and β3 integrin blockade resulted in enhanced invasion in CAOV3 and MONTY1 cells[65]. These latter results were recapitulated in vivo where αvβ3 overexpressing cells displayed a 35% decrease in intra-abdominal metastases and 53% decrease in tumor weight compared to controls[65]. These results highlight the potential mechanistic differences involving integrins between EOC cell adhesion, invasion, and successful secondary tumor formation.

Integrins function in both cell-cell adhesion and binding to basement membrane and ligand components[64]. α2 and β1 integrin subunits contribute to EOC cell adhesion via collagen I facilitating peritoneal attachment and invasion into the mesothelial monolayers[66]. Studies have consistently shown that inhibition of collagen-associated integrins α2β1 lead to attenuated spheroid disaggregation on artificial ECM since primary EOC cells adhere preferentially to type I collagens[54], [62], [67]. Furthermore, Davidson et al. showed high expression of αv and β1 integrin subunits in malignant cells from peritoneal and pleural effusions collected from late-stage EOC patients suggesting a potential role in metastasis[68]. The interaction between vascular cell adhesion molecule 1 (VCAM-1) present on the mesothelium and αvβ1 integrins on EOC cells promotes metastasis and cell migration in xenograft models[69]. Indeed, this study also
demonstrated that the use of function-blocking antibodies against either VCAM-1 or α4β1 integrins show promise in decreasing EOC metastasis[69].

Recent studies suggest that EOC cells may not adhere directly to mesothelial cells, but rather to underlying connective tissue; this is achieved by disrupting cell-cell junctions, a process called mesothelial cell clearance[70]. Iwanicki et al. demonstrated that EOC spheroids use integrin- and talin-dependent activation of myosin traction force to promote mesothelial cell clearance[70]. In this experimental model, mesothelial cell monolayers were plated on fibronectin-coated polyacrylamide gels to mimic physiologically-relevant stiffness of connective tissue. They showed that OVCA433 spheroids induced mesothelial clearance by the above mechanisms[70]. As spheroids promote mesothelial clearance, fibronectin fibrils organized on top of mesothelial cells are redistributed away from between the mesothelium and attaching spheroids[70]. Blocking of α5 integrin, talin 1 and non-muscle myosin II abrogated mesothelial displacement, while ectopic expression of α5 integrin increased myosin-mediated cell spreading, stress fibers, and other cortical actin contractile structures[70]. Collagen I-associated α2 integrin subunit induced fibril reorganization and transmitted traction forces to ECM, but spheroids expressing high levels of α2 integrin rather than α5 integrin were unable to clear the mesothelium[70]. Interestingly, blockade of another fibronectin receptor αvβ3 integrins did not affect mesothelial clearance, suggesting these specific receptors do not have a myosin-contractility roles in EOC metastasis[70]. In a different study, Kokenyesi et al. reported SKOV3 and OVCAR3 cells were unable to invade a collagen I matrix due to their inability to disrupt intracellular interactions, highlighting the importance of integrin-mediated actomyosin contraction to overcome cell-cell attachments[71].

A critical component of tumor invasion is enzymatic degradation of the ECM, which permits cancer cells to penetrate the basement membrane, gain access to the vasculature and successful
formation of secondary tumor growth[40]. Spheroid cell migration using OVCAR5 cells on laminin and collagen IV-coated surfaces showed a modest 2-fold change in cell migration over 24 hours, whereas spheroids on fibronectin and collagen I completely disaggregated to form a monolayer with a 9-fold change in surface area[40], [61]. Addition of an inhibitory antibody against β1 integrin completely eliminated spheroid cell migration on laminin, fibronectin and type-IV collagen, and a 50% reduction on type-I collagen[40], [61]. However, these results suggest that β1 integrin blockade did not prevent initial spheroid attachment, but rather significantly impacted ECM degradation and spheroid disaggregation of invading foci[40]. Overall, β1 integrins partially mediate adhesion of EOC spheroid cells to ECM, but likely plays a more significant role in its degradation and resultant spheroid disaggregation[40], [61].

MMPs are zinc-dependent proteinases that degrade various ECM components, such as collagens, proteoglycans, gelatins, vitronectin, and fibronectin[72]. EOC cells that express higher levels of MMP2 and MMP9 possess increased invasive and metastatic potential[67], [73]. Studies by Kenny et al. demonstrated that contact of EOC cells with mesothelium induces MMP2 expression at the transcriptional and translational levels[74]. Activated MMP2 cleaves various ECM components, including vitronectin and fibronectin, into smaller fragments to improve EOC cell adhesion to αvβ3 and α5β1 integrin receptors[74]. αvβ3 and α5β1 blocking antibodies inhibited cell adhesion, however, this effect was abolished when EOC cells were preincubated with MMP2 antibody[74].

When cells migrate away from the core of an attached spheroid, cell-cell contacts are reduced while adhesion and spreading on ECM occurs[67]. Compared to monolayer culture, MMP2 and MMP9 activities are increased in serum-free medium collected from spheroid culture[67]. Increased α2 integrin and decreased α6 integrin subunits in OVHS1 and HEY spheroids were also
observed[67]. Interestingly, Shield et al. demonstrated OVHS1 and HEY spheroids have reduced disaggregation in the presence of α2, β1 and α2β1 integrin with a coordinated reduction in active MMP2 levels[67].

Furthermore, expression of αvβ6 in EOC is correlated with increased expression and secretion of high molecular weight-urokinase-type plasminogen activator (uPA), pro-MMP2 and pro-MMP9, in tumor-conditioned media[73]. Interestingly, αvβ6 integrin expression is restricted to metastatic EOC cells with little to no expression in benign and normal ovarian epithelial cells[75]. Ahmed et al. suggest that αvβ6 integrin-expressing EOC cell lines have an enhanced capacity to degrade the basement membrane in a plasminogen-dependent manner since this effect was completely abolished by inhibition of uPA, MMP9, and αvβ6 integrins[73].

1.5. Integrins as therapeutic targets for EOC

Novel therapies that focus on malignant cells and the tumor microenvironment in EOC have gained substantial interest due to the heterogeneity of the disease. As described in detail above, integrins are key regulators at various steps in the unique metastatic cascade of this disease, particularly in spheroid formation, and for peritoneal invasion where integrin-ECM interactions are essential for initiating spheroid adhesion[36]. Insight into the cellular mechanisms involved in cancer cell survival and progression over the last decade have led to the development of integrin inhibitors[13]. Preclinical and clinical studies of integrin antagonists show promising results in effectively blocking tumor progression[76].

Integrin inhibitors represent a feasible therapeutic strategy since the majority of Phase I clinical trials demonstrate that these agents are well-tolerated by patients in conjunction with cytotoxic chemotherapy or radiotherapy[13], [36]. The chimeric monoclonal antibody
Volociximab targets $\alpha_5\beta_1$ integrin and has been successful in inhibiting angiogenesis and impairment of tumor growth[77]. Preclinical data shows that intraperitoneal treatment of SKOV3ip1 xenografted mice with Volociximab reduced tumor burden and ascites accumulation by 83% and 97%, respectively[77]. Encouragingly, clinical trials showed that EOC patients with platinum-resistant disease treated with a weekly administration of Volociximab was well tolerated[78]. ATN-161 is a non-RGD based synthetic pentapeptide derived from fibronectin that binds to and blocks both $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins[79]. This agent has shown promise using mouse xenograft models of breast cancer metastasis and it was safe in patients with stable disease in Phase I trials, but has not been tested in EOC patients yet[80], [81]. Etaracizumab is an anti-human monoclonal antibody against $\alpha_v\beta_3$ developed after encouraging preclinical results showing decreased tumor burden in SKOV3ip1 and HEYA8 xenograft mouse models[82]. However, clinical trials showed minimal effectiveness as a therapeutic treatment for metastatic disease[79]. Another humanized antibody Intetumumab targeting $\alpha_v\beta_3$ and $\alpha_v\beta_5$ showed effective inhibition of cancer cell adhesion and migration of six different uterine serous papillary carcinoma cell lines in vitro[83]. Phase I clinical trials show that it is safe, it localized to tumors, and shows signs of potential anti-tumor activity, but these early findings require additional trials[84]. Although promising results have been seen with anti-integrin $\alpha_v\beta_3$ antibodies, results from Kaur et al. suggest that of $\alpha_v\beta_3$ overexpression in fact correlates with favorable patient outcome; thus, further clinical investigations are required[65].

Although approaches for targeting integrins may offer therapeutic potential in the future, no single integrin receptor complex inhibition strategy has shown sufficient clinical trials results to progress for further investigation yet[36]. One major hurdle impeding success may be the complexity and dynamics of integrin functions implicated in EOC tumor growth and metastasis.
(Figure 1.2). For instance, the crucial interaction of EOC cells for adhesion via fibronectin is not limited to $\alpha_5\beta_1$ integrin, as $\alpha_5\beta_3$ or $\alpha_3\beta_1$ integrins can compensate for loss of function[36]. Adding to this challenge will be the broad intratumor and interpatient heterogeneity in this disease. Taken together, we foresee that a combined approach of targeting multiple integrin-associated pathways may be worthy of future exploration in both experimental and clinical applications.
Figure 1.2
A schematic model of ovarian cancer dissemination and the role of integrins in cancer metastasis. The primary site of origin for HGSOC is the secretory epithelial cells of the distal fallopian tube from precursor STIC lesions and the increase in L1CAM and increased ligation of fibronectin to $\alpha_5\beta_1$ promotes cell detachment. Detached EOC cells survive in hypoxic and anchorage-independent conditions by forming heterogenous multicellular structures known as spheroids to avoid anoikis. Spheroid formation can be enhanced with the interaction of integrins with various ECM proteins. Integrins such as $\alpha_2\beta_1$ and $\alpha_3\beta_1$ clustering leads to the loosening of intercellular adhesions between cells and contributes to EMT-MET switching. The final step of EOC metastasis occurs when spheroids penetrate mesothelial surfaces, and integrin-mediated degradation of the ECM within the basement membrane underlying the peritoneum leads to secondary metastasis. [created by biorender.com]
1.6. **The role of β5 Integrin in cancer pathogenesis**

Integrin β5 (ITGβ5) encodes for an integrin subunit that interacts with the integrin αv to form a functional transmembrane heterodimer[13]. It is present on the surface of several cancer subtypes and interacts with ligands with the arginine-glycine-aspartate (RGD) sequence motif[13, 92]. Although no selective ligand has been identified for integrin β5, it is less promiscuous than other integrins and binds preferentially to vitronectin and most small molecules that bind to the heterodimer αvβ3 [93, 94]. Previous studies have shown that integrin β5 is a prognostic bio-marker within various malignancies including human pancreatic, breast, gastric and ovarian cancers [95–98]. Although, a new study aimed to explore the prognostic values of the integrin superfamily in HGSOC using The Cancer Genome Atlas (TCGA) and GSE9891 dataset found that Integrin β5 was significantly downregulated in HGSOC compared to control groups [92].

There has been accumulating evidence of β5 integrin facilitating cell survival, angiogenesis, cancer cell migration, invasion and transforming growth factor β (TGF-β) induced EMT [58, 100, 101]. Hood et al. show that αvβ5 and αvβ3 contribute to sustained Ras-extracellular signal-related kinase (Ras-ERK) signaling in blood vessels, which is a requirement for endothelial cell survival and angiogenesis [93]. In Glioblastoma, integrin β5 overexpression is not only associated with poor patient survival but also promotes migration and invasion in glioma cells [94]. Furthermore, there is evidence that integrin β5 plays a role in carcinoma cell motility through the binding of PAK4, a p21-activated group II kinase, to the cytoplasmic domain of integrin β5 [95]. The group show that the engagement of integrin β5 and vitronectin led to the redistribution of PAK4 from cytosol to colocalization with integrin β5 and ultimately
human breast carcinoma cell migration [95]. The depletion of integrin β5 in triple-negative breast carcinoma cells reduces the tumor take, growth and angiogenesis, whereas the re-expression of integrin β5 can rescue this phenotype [89]. Bianchi et al. revealed that integrin β5 mediates Src-FAK and MEK-ERK signaling events and inhibition of these pathways produces the same phenotype as integrin β5 deficiency [89]. Interestingly, integrin β5 is associated with enhanced cell glycolysis to promote cancer cell growth and proliferation and counteracting cisplatin cytotoxicity in breast and cervical cancer cells [96]. Wang et al. demonstrated that Src-induced phosphorylation of FAK at Tyr861 was involved in integrin β5-mediated glycolysis, further expanding the different functions of integrin β5—FAK signaling [96].

TGF-β signaling is critical for various functions such as apoptosis, cell proliferation, differentiation, adhesion, tumor progression and the promotion of EMT [104–107]. Using mRNA sequencing data and clinical prognostic information of hepatocellular cancer patients from TCGA database, integrin β5 was identified as EMT associated gene and part of a 5-gene prognostic risk model for patients [101]. In colorectal cancer, Shi et al. show that higher integrin β5 expression is associated with EMT process and TGF-β signal activation [102]. When integrin β5 was silenced, the EMT process was attenuated because there was reduced Smad phosphorylation by the decrease in Snail1, Twist1 and TGFβ1 [102]. These results suggest that the upregulation of integrin β5 may possibly enhance TGF-β signaling and the EMT process, thereby promoting tumor growth and metastasis in colorectal cancer [102]. These findings are consistent with previous reports that show the loss of integrin β5 hinders breast carcinoma cell growth, tumor angiogenesis and migration by inhibiting Src/FAK and MEK/ERK signaling [89]. Bianchi et al. highlights the upregulation of integrin β5 during TGF-β induced EMT in breast carcinoma that requires the Smad transcription factors [58]. The depletion of integrin β5
significantly reduced the invasiveness of breast carcinoma cells, blocked the TGF-β induced EMT and therefore impairing adhesion to cell-matrix and integrin signaling [58]. Although it has been shown that intact TGF-β signaling is critical to control EMT in EOC ascites-derived spheroids and promotes the malignant characteristics of these structures, the relationship between TGF-β and integrin β5 in ovarian cancer is unclear [57].

While there is a body of literature that suggests roles of integrin β5 in various malignancies, the function of integrin β5 within ovarian cancer is limited. Gillan et al. suggest that Periostin (PN), formerly called osteoblast-specific factor-2, is secreted by EOC cells, accumulates in the ascites and functions as a ligand for integrins αvβ3 and αvβ5 to promote EOC dissemination, cell adhesion and migration [103]. They show that purified recombinant PN supports EOC cell adhesion that can be inhibited by a monoclonal antibody against αvβ5 and αvβ3 but not anti-β1 [103]. Moreover, Maubant et al. derived a cisplatin-resistant cell line, IGROV-R10, and when grown in monolayer culture, they have an enhanced ability to spontaneously release cell clusters with high proliferative abilities [104]. Compared to the parental IGROV1 cells, IGROV-R10 cells show a strong enrichment of αvβ5 on the surface of monolayer cells as well as the spontaneously formed cell clusters in suspension [104]. This highlights the chemoresistance associated dysregulation of integrin expression on cell surfaces and is the first report to access the altered expression of integrin β5 due to cisplatin resistance [104].

Furthermore, integrin β subunit activate a common set of cytoplasmic tyrosine kinases such as FAK, a gene that is amplified by 24% in serous ovarian cancer and is associated with decreased overall patient survival [76, 112]. Tancioni et al. show that the inhibition of FAK or integrin β5 knockdown reduce ovarian tumor cell growth under anchorage-independent conditions, which corresponds to decreased orthotopic tumor growth [91]. It seems that FAK inhibition disrupts the
autocrine or paracrine signaling that regulates integrin β5 in addition to osteopontin levels in ovarian carcinoma cells [91].

1.7. Role of LKB1-NUAK1 signaling in EOC Tumor Progression

The revelation of important molecular pathways in spheroid formation would increase understanding of metastasis and potentially uncover novel therapeutic targets. Liver Kinase B1 (LKB1) is encoded by the STK11 gene and is a serine-threonine master kinase[106]. It is widely expressed in established ovarian cancer cell lines and patient derived ascites[107]. LKB1 expression increases in ovarian cancer spheroids compare to monolayer, suggesting an important role in spheroid formation [107]. Our group has shown that LKB1 is critical for metastasis and the loss of LKB1 can decrease anchorage-independent growth and viability of spheroids[108]. LKB1 is activated when it forms a complex with accessory proteins STRAD and MO25 [109]. LKB1 can then activate downstream target AMP-activated protein kinase (AMPK), which regulates metabolic stress through phosphorylation modification of threonine 172 [109]. This results in the coordinated downregulation of anabolic pathways and upregulation of catabolic pathways in order to create homeostasis [109]. Our group went on to show that LKB1 ablation resulted in significantly decrease number of viable cells and increased dead cell count in spheroids culture of EOC cell lines tested [108]. This highlights the tumor forming and metastatic potential of EOC cells by the loss of LKB1. However, our group has shown that LKB1 pro-metastasis role in ovarian cancer occurs through AMPK-independent signaling because in spheroids lacking LKB1, p-AMPK levels were maintained [108]. This suggests that the ability of LKB1 to regulate 12 other AMPK related kinases may represent other targets by which LKB1 maintains spheroid formation and cell viability.
One of the top substrates identified by multiplex inhibitor beads-mass spectrometry analysis from the loss of LKB1 is NUAK1 [38]. NUAK1 can function in tumor progression through regulating apoptosis, invasion and metastasis in tumors [110]. For instance, loss of NUAK1 led to a reduction of ATP levels and decreased proliferation in hepatocellular and pancreatic carcinoma cells [111]. In ovarian cancer specifically, NUAK1 overexpression has been linked to lower progression free survival and lower overall survival [112]. Furthermore, there is an increased risk of advanced stage diagnosis and reoccurrence after cytoreduction surgery [112]. NUAK1 has increased expression in spheroids compared to monolayer and is involved in increasing EOC cell adhesion [38]. By examining NUAK1 Knock out (KO) green fluorescent protein (GFP) labeled spheroids, we were able to evaluate spheroid formation. It was observed that the loss of NUAK1 creates spheroids that are less compact and with an accumulation of dead cells around the periphery [38]. NUAK1 appears to impair single cell adhesion and spheroid formation. We postulate that NUAK1 regulates cell adhesion and ECM interactions to help form spheroids that spread through the peritoneal cavity. Through Gene Set Enrichment analysis (GSEA), the hallmark database revealed multiple cell attachment pathways involved in integrin cell attachment that were differentially expressed in NUAK1 knockout spheroids [38]. The FN1 gene encoding fibronectin, a known critical ECM protein in EOC spheroids, showed a 745-fold decrease in the NUAK1 knockout spheroids [38]. When comparing OVCAR8 parental monolayer cells to spheroids, there was an increase in fibronectin expression with the presence of multiple isoforms [38]. However, in OVCAR8-NUAK1KO spheroids, there was no detectable expression of fibronectin, suggesting NUAK1 may be critically required for the expression of fibronectin. Interestingly, when soluble fibronectin was reintroduced to NUAK1KO spheroids, native spheroid formation was rescued [38]. Altogether, this revealed a
new mechanism through which NUAK1 promotes EOC cell adhesion and spheroid compaction through fibronectin matrix production.

1.8. Research goal, hypothesis and objectives

Our previous studies show that the ablation of LKB1-NUAK1 signaling pathway in EOC cells resulted in a substantial loss of fibronectin expression leading to spheroid disaggregation [38]. Numerous studies have shown that fibronectin binds to its canonical receptor $\alpha_5\beta_1$ in ovarian cancer but there was no differential expression of this receptor [6], [41]. Our transcriptome analysis demonstrated a coordinated reduction in $\beta_5$ integrin expression due to NUAK1 loss [38] and therefore, I sought to investigate the function of $\beta_5$ integrin in ovarian cancer. I hypothesize that $\beta_5$ integrin is required for epithelial ovarian cancer cell adhesion, spheroid formation and subsequent spheroid reattachment. To test this hypothesis, I characterized the mRNA expression of $\beta_5$ integrin using publicly-available databases and protein expression using western blot analysis of established and patient ascites-derived EOC cell lines. Furthermore, I performed siRNA-mediated knockdown in established cell lines in monolayer and anchorage independent culture to assess the functional role of $\beta_5$ integrin using cell adhesion, cell viability, spheroid viability and reattachment assays. This project has allowed me to elucidate the role of $\beta_5$ integrin in ovarian cancer cell adhesion and spheroid reattachment which have not previously been explored.
1.9. References


[19] P. Dr. Nicolas Rodondi, MD, MAS, Ms. Wendy P. J. den Elzen, MSc, Dr. Douglas C. Bauer, MD, Dr. Anne R. Cappola, MD, ScM, Dr. Salman Razvi, MD, FRCP, Dr. John P. Walsh, MBBS, FRACP, PhD, Dr. Bjorn O. Åsvold, MD, PhD, Dr. Giorgio Iervasi, MD, Dr. Misa Imaizumi, “Transformation of the Fallopian Tube Secretory Epithelium Leads to High-grade Serous Ovarian Cancer in Brca;Tp53;Pten Models,” *Bone*, vol. 23, no. 1, pp. 1–7, 2010, doi: 10.1038/jid.2014.371. doi:10.1038/jid.2014.371.


[100] M. Pickup, S. Novitskiy, and H. L. Moses, “The roles of TGFβ in the tumour


doi:10.1158/2159-8290.CD-17-0533.


http://www.nature.com/articles/nprot0915-1457b.


[119] J. G. Lock et al., “Reticular adhesions are a distinct class of cell-matrix adhesions that


Chapter 2

2. Materials and Methods

2.1. Expression Analysis using Publicly Available Datasets

To obtain the expression level of ITGB5 and ITGB8 we downloaded RNAseq TPM gene expression data for protein coding genes RSEM from Depmap (Source: DepMap, Broad (2021): DepMap 21Q2 Public. figshare. Dataset. (https://doi.org/10.6084/m9.figshare.14541774.v2). Counts are Log\(^2\) transformed, using a pseudo-count of 1. Prior to downstream analysis dataset was filtered by removing all samples that were not identified as 'Ovarian Cancer' in 'disease' column. RNAseq data was downloaded from cBioportal using all integrin gene names as query search. The provisional ovarian serous carcinoma dataset for solid tumour data (n=606) was used and the 47 ovarian cancer cell lines from the Cancer Cell Line Encyclopedia data was used.

2.2. Cell culture and treatments

OVCAR3, OVCAR5, OVCAR8, CAOV3, HEYA8, COV362, OVCA 420 and OVCA 433 cells were cultured in RPMI-1640 (Wisent, St. Bruno, QC). FT190, TOV21G, ES-2, 105C, KOC7C, OVTOKO, OVMANA, SMOV2, and the patient ascites-derived (iOvCa) cells were cultured in DMEM-F12 (Life Technologies). For all cell lines growth medium was supplemented with 10% FBS (Wisent, St. Bruno, QC). OVCAR3, OVCAR5, OVCAR8, CAOV3, HEYA8 cells were obtained from ATCC. COV362 cells were received from Z. Khan (University of Western Ontario, London, ON). The immortalized human fallopian tube secretory epithelial cell line FT190 was provided by R Drapkin (University of Pennsylvania, Philadelphia, PA). The iOvCa cell lines were established by Dr. Gabriel DiMattia (University of Western Ontario, London, ON) based on a protocol described previously [113]. TOV21G was provided by Dr. Anne-Marie Mes-Masson (University of Montréal, Montreal, QC) and ES-2, OVCA 420 and OVCA 433 are from Dr. Barbara Vanderhyden (University of Ottawa, Ottawa, ON). The 105C originally referred to as SCHM-1 were obtained from Dr. Hal Hirte (McMaster University, Hamilton, ON). KOC7C, OVTOKO, OVMANA and SMOV2 were kindly provided by Dr. Hiroaki Itamochi (Iwate Medical University, Iwate-ken, Japan).
Cells were grown in a humidified incubator at 37°C with 5% CO₂. Adherent cells were cultured on tissue cultured-treated polystyrene (Sarstedt, Newton, NC). Spheroids were formed by culturing cells on ultra-low attachment dishes (Corning, NY) that have a hydrophilic and neutral coating to allow for cell clustering as described previously [33].

2.3. Small-interfering RNA Transfection

Cells were seeded in 6-well dishes and seeding density per well was based on proliferation of each cell line. FT190 cells were seeded at a density of 3.0 x 10⁵ cells/well, OVCAR8 1.25 x 10⁵ cells/well, HEYA8 1.5 x 10⁵ cells/well, TOV21G 2.0 x 10⁵ cells/well, and ES-2 1.0 x 10⁵ cells/well. After 24 hours, cells were transfected according to the manufacturer’s protocol (Dharmacon, Thermo Fisher Scientific). The combination of 4 μL DharmaFect-1 (T-2001-02) with 10 nM of siRNA in 2 ml of media was incubated for 30 minutes. ITGβ5 (L-004125-00-0005) and non-targeting control pool (D-001206-14-05) siGENOME SMARTpool siRNAs were obtained from Dharmacon. The combined siRNA/DharmaFect1 complexes were added to each well containing cell and incubated at 37°C with 5% CO₂. After 24 hours, the transfection media was replaced and 72 hours post transfection, cells were trypsinized and seeded for adherent and spheroid culture for experimental conditions. Lysates were also collected 3 days post-transfection for western blot analysis.

2.4. Immunoblot analysis

Whole cell lysates from were obtained from 3 days of adherent culture or 3 days post transfection. Cells were collected by washing the wells with PBS and scraping cells in lysis buffer [50mM HEPES pH7.4, 150 mM NaCl, 10% glycerol, 1.5 mM MgCl₂, 1 mM 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, QC), and 225mM β-glycerophosphate]. Protein concentration was determined with Bradford assay (Bio-Rad Laboratories, Hercules, CA).

Protein samples were prepared using 30 μg of protein determined by Bradford assay and resolved by SDS-PAGE using 6% or 8% gels for ~ 2.5 hours. Electrophoresis was followed by a
1 hour wet-transfer onto PVDF membrane (Immobilon-P) at 100 V. Membranes were blocked for 1 hour with 5% Bovine Serum Albumin (BSA) diluated in TBST (10mM Tris–HCl, pH 8.0, 150mM NaCl and 0.1% Tween-20). Primary antibody against ITGβ5 (1:1000 dilution in 5% BSA, Cell signaling #3629), Tubulin (1:20 000 dilution in 5% BSA, Sigma T5168) and Vinculin (1:20 000 dilution in 5% BSA, Sigma V9264) was left on membranes overnight at 4°C. Next day, membranes were washed 3x with TBST for a total for 45 minutes. Membranes were then incubated for 1 hour with peroxidase-conjugated anti-rabbit (1:10 000 dilution in 5% BSA, Sigma NA934V) or anti-mouse (1:10 000 dilution in 5% BSA, Sigma NA931V) antibodies and washed 3x for 45 minutes with TBST. The membranes were then incubated with Immobilon Classico Western HRP Substrate (Millipore Sigma (Oakville, ON) for 5 minutes and images were captured using the ChemiDoc™ Imaging system (Bio-Rad) and bands were quantified using Image Lab 4.1 software.

2.5. Flow Cytometry

Three days following siRNA transfection, cells were detached using 0.53mM EDTA (Wisent Bioproducts 325-060-EL) and placed in the incubator at 37°C with 5% CO₂ for 10 minutes, cells were mechanically dislodged with a P200 pipette and incubated again for 10 minutes. Once cells are dissociated, 2 mL of FACS Buffer (5% FBS diluted in PBS) was added and cells were counted (TC10 cell counter, Bio-Rad) to ensure 5 x10⁵ cells were transferred to 1.5 mL Eppendorf tubes. Samples were centrifuged at 2000rpm for 5 minutes and washed with 150 μL FACS Buffer. After 2-3x washes and centrifuged, 200 μL of FACS Buffer was added and incubated with primary AlexaFluor488 αβ₅ anti-human/rat (5 μL, Biolegend) antibody for 1 hour. Samples were washed 2-3x with FACS Buffer and on the last wash, add 50 μL with fixation Buffer (2% paraformaldehyde diluted in PBS) for 20 minutes. After the samples were centrifuged, 200 μL of FACS Buffer was added and samples were incubated overnight at 4°C. Next day, samples were centrifuged and washed 2-3x and 500 μL volumes were strained and transferred over to flow tubes. Samples were analyzed using an EPICS XL-MCL or Cytomics FC500 flow cytometer (Beckman Coulter).
2.6. **Timed Cell Adhesion Assay**

Cells were seeded in a 24-well standard tissue culture-treated dish at 200,000 cells per well. At specific time points determined from previous experiments (Collins & Shepherd, unpublished) (45 minutes for HEYA8 cells, 4 hours for OVCAR8 cells, 1 hour for FT190, TOV21G and ES-2 cells), non-adherent cells were aspirated, the plate was washed with PBS, then trypsinized and counted with Trypan Blue to quantify single cell adhesion. Time points were determined using a time course cell adhesion assay, where single cell adhesion was quantified using Trypan Blue for all 5 cell lines without transfection. The time points used were: 30 min, 45 min, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours and 6 hours. Cells were counted using TC10 cell counter (Bio-Rad) and values were normalized to the initial seeding density. The time point at which all cells have attached was used as the time point comparing siNT and siITGβ5 conditions for each cell line.

2.7. **Determination of Doubling Time**

Three days after siRNA transfection cells were seeded in a 48-well adherent culture at a density of 7500 cells per well. (Essen Bioscience, Ann Arbor, MI), Percent confluence was measured by Incucyte for 7 days by capturing phase contrast images every 3 hours and taking the average of 9 images. To calculate doubling time, an exponential growth curve was fitted to the confluence-over-time results in Graphpad PRISM.

2.8. **Trypan Blue Exclusion Viability Assay**

Three days following siRNA transfection cells were seeded in a 24-well standard tissue culture-treated plate at a density of 125,000 cells per well or ultra-low attachment (ULA) plates at a density of 50,000 cells per well for 24 or 72 hours. Adherent cells were detached using 50 μL trypsin-EDTA (0.25%) and incubated at 37°C with 5% CO₂ for 10 minutes. After adding 100 μL of FBS, cells were transferred to 1.5 mL Eppendorf tubes and 150 μL of Trypan Blue dye (ThermoFischer Scientific) was added to each tube. Stained cells were added to the cell counter slide and viability readings were taken using TC10 cell counter (Bio-Rad).

Spheroids from 24-well ULA plates were transferred to 1.5mL Eppendorf tubes and centrifuged at 1500rpm for 3 minutes. Media was completely aspirated from tubes avoiding the pelleted
spheroids at the bottom. Pellets were washed twice with 1mL of PBS, centrifuged again and media was aspirated. A volume of 50 μL of trypsin-EDTA (0.25%) was added and tubes were placed in 37°C water bath for 30 minutes with gentle vortex every 10 minutes. After incubation, 100 μL FBS was added to resuspend the dissociated spheroids followed by resuspending in 150 μL of Trypan Blue dye (ThermoFischer Scientific). Once cells were counted using TC10 cell counter, the total cell number and live cell number were recorded.

2.9. CellTiter-Glo Spheroid Viability assay

Three days after siRNA transfection cells were seeded in 96-well ULA plate at a seeding density of 4000 cells per well for 24 or 72 hours. CellTiter-Glo® reagent (Promega) was added to each well at a volume of 100 μL (1:1 ratio of CellTiter-Glo® reagent and Media in the well). Three wells for each time point consisted of only media and the CellTiter-Glo® reagent at a 1:1 ratio (Blank controls). Plates were incubated overnight at -80°C to ensure lysis. The following day 198 μL was transferred from each well into a white-walled 96-well plate. Readings were measured on Wallac 1420 victor 2 spectrophotometer plate reader, measuring luminescence. The average luminescence readings for Blank control wells (i.e., media alone) were subtracted from the luminescence readings from the average per experimental condition.

2.10. Spheroid Reattachment Assays

Three days following siRNA transfection cells were seeded in 96-well ULA plates at a seeding density of 4000 cells per well for 24 or 72 hours. The cultured spheroids were then transferred to 48-well adherent culture plates with 1 mL of 10% FBS media and incubated at 37°C with 5% CO₂. Spheroids were permitted to reattach and disperse for an additional 24 hours prior to fixing and staining using HEMA3 (Fisher, Kalamazoo, MI, USA). Spheroid reattachment and dispersion areas were calculated using the Trainable Weka segmentation analysis plugin on the Fiji Image J 2.1 software (NIH). Total spheroid reattachment for full wells was quantified by creating classifiers that can differentiate between the background well and the stained area of the reattached spheroid. Once the program has differentiated the image using the classifiers, a black and white binary image was created in order for the program to calculate the total area for the regions of interest. Spheroid dispersion area was calculated using the same Trainable Weka segmentation analysis plugin on the Fiji Image J 2.1 software (NIH) using high magnification
images. Classifiers were used to differentiate between the background and the stained spheroid area and a binary image was created to calculate the total spheroid reattachment area for single spheroids. This process was repeated using 3 different classifiers to differentiate between spheroid core, dispersing monolayer and well background area and calculate the area for the reattached spheroid core. Spheroid dispersion was defined as the spheroid core area subtracted from the total reattached spheroid area for OVCAR8, TOV21G and ES-2. Due to the morphology of the spheroids of FT190 and HEYA8, this method could not be used. Instead, total reattached spheroid area was quantified alone.

Spheroid reattachment assays with the ECM-coated plates were completed with spheroids cultured in 96-well ULA plates at a seeding density of 4000 cells per well for 24 hours. Spheroids were then transferred to either BSA, fibronectin or vitronectin coated 24-well adherent culture plates with 1 mL of 0% FBS media and incubated at 37°C with 5% CO2. The fibronectin-coated plates were acquired from Corning Life sciences and stored at 4°C until use. The vitronectin-coated plates were prepared using human recombinant vitronectin (Millipore Sigma, SRP3186) as per the manufacturer’s guidelines. The BSA-coated plates were prepared by 0.1% BSA diluted in sterile water. Spheroids were permitted to reattach and disperse for an additional 24 hours prior to fixing and staining using HEMA3. Spheroid reattachment and single spheroid areas were calculated using the Trainable Weka segmentation analysis plugin on the Fiji Image J 2.1 software (NIH).

2.11. Statistical Analysis

Statistical analysis was completed using Graphpad PRISM 9 (GraphPad Software, San Diego, CA). Analyses were performed using two-tailed Student’s t-test and a p-value less than 0.05 was considered statistically significant (*, p < 0.05; ***, p < 0.01; ****, p < 0.001).
2.12. References


Chapter 3

3. Results

3.1. Integrin gene expression in serous ovarian tumours and ovarian cancer cell lines

To begin, mRNA expression levels of all integrins were evaluated using publicly available datasets from the Cancer Genome Atlas (TCGA). By assessing the expression of the superfamily of integrins among ovarian cancer tumors and cell lines, we can then evaluate the expression of integrin β5 within this framework. The data suggests that there is wide variability in expression of both integrin α- and β-subunits (Figure 3.1). Interestingly, the expression of integrin β5 is high relative to other β-subunits in both serous ovarian tumours and cell lines. Integrin β5 mRNA expression is the third highest β-subunit in the tumours (Figure 3.1A) and second highest in gene expression among ovarian cancer cell lines (Figure 3.1B). Furthermore, the α-subunit associated with integrin β5 to form a functional heterodimer, integrin αv also has relatively high expression and is the second highest α-subunit expressed in both serous ovarian tumors (Figure 3.1A) and ovarian cancer cell lines (Figure 3.1B).

3.2. Integrin β5 is expressed across established ovarian cancer cell lines and patient-ascites derived cell lines

To further validate the data from TCGA, we sought to evaluate the expression of integrin β5 within well-established cell lines across two subtypes of ovarian cancer: High-grade serous ovarian cancer cell lines (HGSOC) and clear cell carcinoma cell lines (OCCC). HGSOC is the most common and most aggressive subtype accounting for 75% of all EOCs [8]. Although early detection of OCCC has a patient 5-year disease-free survival rate of 86-89%, advanced stage prognosis is remarkably poorer than that of patients with serous carcinoma [114]. The use of readily available and well-established cell lines from both subtypes provides appropriate context for assessing the role of integrin β5 in EOC. Transcript expression data for HGSOC and OCCC was obtained from the Cancer cell line encyclopedia (CCLE) dataset and the protein expression was evaluated using western blot analysis with adherent culture day 3 lysates.
The mRNA expression of integrin β5 is expressed with high variability across multiple HGSOC and clear cell carcinoma cell lines (Figure 3.2A). When assessing the protein level expression of integrin β5, it was evident that integrin β5 expression varies across both ovarian cancer subtypes but is still detectable (Figure 3.2B, 3.2C). The clear cell carcinoma cell lines that have shown the highest integrin β5 protein expression level were OVMANA and KOC7C whereas, the SMOV2, TOV21G, ES-2 and 105C show similar yet lower levels (Figure 3.2B, 3.2C). The HGSOC cell lines that show the highest integrin β5 protein expression are the OVCA 420, OVCA 433 and OVCAR5 cell lines (Figure 3.2B, 3.2C). The CAOV3, HEYA8 and OVCAR8 cell lines show similar integrin β5 protein expression to the normal fallopian tube immortalized control cell line, FT190 (Figure 3.2B, 3.2C). The immortalized fallopian tube cell line is used as a control since it is now widely considered that HGSOC initiates from the secretory epithelial cells of the distal fallopian tube [26]. The HGSOC cell lines that show very little integrin β5 protein expression are the OVCAR8 and COV362 (Figure 3.2B, 3.2C).

Several early-passage ovarian cancer patient-ascites derived cell lines generated by our laboratory (G. DiMattia) were also used to evaluate the protein expression of integrin β5 through western blot analysis. Similar to the established cell lines, the patient-ascites derived cell lines exhibit detectable integrin β5 protein expression (Figure 3.2D, 3.2E). This overall assessment of integrin β5 expression among established cell lines enabled us to identify appropriate cell lines for functional assays using targeted siRNA-mediated knockdown. The cell lines chosen display a spectrum of integrin β5 expression to account for the differences across cell lines when evaluating the function of integrin β5 within EOC (Table 3.1).

### 3.3. Transient Knockdown of Integrin β5 leads to a decrease in cell viability of EOC cells in adherent culture

To evaluate the role of integrin β5 within EOC, siRNA-mediated knockdown of ITGβ5 in OVCAR8, HEYA8, TOV21G and ES-2 cell lines was completed (Figure 3.3). OVCAR8 and HEYA8 are well established HGSOC cell lines and TOV21G and ES-2 are representative OCCC cell lines. Based on the previous western blot analysis, HEYA8 have higher protein expression of integrin β5 than OVCAR8 (Figure 3.2B, 3.2C). Whereas, both TOV21G and ES-2 show similar protein level expression of integrin β5 (Figure 3.2B, 3.2C). Transient knockdown was also performed on the FT190 cell line as a control for HGSOC (Figure 3.3). The knockdown of
Figure 3.1: Integrin β5 has relatively high expression across serous ovarian tumors and cancer cell lines (A) Integrin family gene expression in serous ovarian tumors and ovarian cancer cell lines (B) obtained from publicly-available The cancer genome atlas (TCGA) dataset. Integrin αv has the second highest alpha subunit gene expression in ovarian tumors and cancer cell lines. The shading represents the subclasses of I-domain and non-shaded represent non-I domain within α-subunits.
Figure 3.2: Integrin β5 transcript and protein expression is detectable across two EOC subtypes and patient ascites derived cell lines

(A) Transcript expression (transcript per million) was obtained from Cancer Cell line encyclopedia (CCLE) dataset for clear cell carcinoma and HGSOC cell lines. (B) Representative western blots of integrin β5 protein expression across both clear cell carcinoma and HGSOC cell lines. The results are presented as the mean±SD. (n=3) (C) Densitometry analysis shows pixel intensity volume for integrin β5 relative to vinculin across both clear cell carcinoma and HGSOC cell lines. (D) Representative western blot of integrin β5 across patient ascites derived iOVCA cell lines. (E) Densitometry analysis shows pixel intensity volume for integrin β5 relative to tubulin. The results are presented as the mean±SD. (n=3).
Table 3.1 Summary\(^a\) of Epithelial ovarian cancer cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>EOC subtype</th>
<th>Mutations</th>
<th>Integrin Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>FT190</td>
<td>Immortalized normal distal fallopian tube</td>
<td>--</td>
<td>Increased L1CAM expression leads to increased spheroid formation due to the upregulation of integrin subunits (\alpha_5) and (\beta_1) [3]</td>
</tr>
<tr>
<td>OVCAR8</td>
<td>High grade ovarian serous adenocarcinoma</td>
<td>CTNNB1, ERBB2, KRAS, TP53</td>
<td>Increased L1CAM expression leads to increased spheroid formation due to the upregulation of integrin subunits (\alpha_5) and (\beta_1) [3]</td>
</tr>
<tr>
<td>HEYA8</td>
<td>High grade ovarian serous adenocarcinoma</td>
<td>BRAF, KRAS</td>
<td>Treating with integrin subunit (\beta_1) blocking antibody MAB13 leads to spheroid disaggregation [4]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Increased (\alpha_2) and decreased (\alpha_6) integrin subunit expression was observed in spheroids [5]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>The use of anti-(\alpha_5\beta_3) Etaracizumab leads to decreased tumor burden in xenograft mouse model [6]</td>
</tr>
<tr>
<td>TOV21G</td>
<td>Ovarian clear cell adenocarcinoma</td>
<td>KRAS, PIK3CA, PTEN</td>
<td>--</td>
</tr>
<tr>
<td>ES-2</td>
<td>Ovarian clear cell adenocarcinoma</td>
<td>BRAF, PALB2, TERT, TP53</td>
<td>--</td>
</tr>
</tbody>
</table>

\(^a\)Data presented in table obtained for Cancer cell line encyclopedia (CCLE) dataset for information on subtype and mutations.
ITGβ5 was initially validated through western blot analysis using an antibody against integrin β5 (Figure 3.3A). As a method of additional validation, flow cytometry using an antibody for the heterodimer αvβ5 was performed which shows that knocking down ITGβ5 leads to an 80-85% decrease in the expression of the functional heterodimer (Figure 3.3B, 3.3C). Our results suggest FT190, HEYA8, ES-2 and TOV21G have a higher level of knockdown than OVCAR8 (Figure 3.3B, 3.3C). This could be attributed to the already low expression of integrin β5 in the OVCAR8 (Figure 3.2B, 3.2C).

Cell viability assays were completed to assess the viability of knockdown in adherent conditions. To evaluate the viability of cells, Trypan blue exclusion counting was conducted at both 24 and 72 hours. The results indicate that ITGβ5 knockdown significantly decreased the number of viable cells in adherent culture for FT190 at 24 hours and OVCAR8 cells at both 24 and 72 hours (Figure 3.3D, 3.3E). Although no significant difference in viability is detected in OCCC cell lines TOV21G and ES-2 at 24 hours, there is a significant decrease in cell viability at 72 hours (Figure 3.3D, 3.3E). Interestingly, the HEYA8 cells showed no significant difference in relative cell viability at 24 hours but by 72 hours, there was a significant increase in the number of viable cells (Figure 3.3D, 3.3E). Altogether, it seems that the loss of integrin β5 impacts cell viability in adherent culture as early as 24 hours in culture.

3.4. Integrin β5 knockdown decreases cell adhesion in EOC without altering cell proliferation

Integrins pose as critical regulators of cancer cell adhesion therefore we sought to evaluate the role of integrin β5 as a cell adhesion receptor in EOC. Cell adhesion assay was quantified in adherent culture at an appropriate time as determined by time course analysis for each cell line. Single cell adhesion was quantified using Trypan blue cell counting. The loss of integrin β5 in FT190, OVCAR8 and TOV21G shows a significant decrease in the ability of these cells to attach to standard tissue culture-treated substratum (Figure 3.4A, 3.4B). Interestingly, integrin β5 knockdown appeared to promote OVCAR8 cells to form spontaneous cell clusters rather than adhere to the tissue culture plastic. This phenotype was also evident in the other HGSOC cell line, HEYA8 but to a lesser degree. There is no significant change in the ability of HEYA8 and ES-2 cells to attach when ITGβ5 is knocked down (Figure 3.4A, 3.4B). To assess whether the
altered adhesion affects proliferation rate, cell doubling time analyses were completed. The results indicate that there is no significant change in doubling times between the control and knockdown conditions among all cell lines (Figure 3.4C). Overall, the results suggest that the knockdown of ITGβ5 decreases the cell adhesion of FTE cells and the majority of EOC cell lines.

3.5. Transient knockdown of Integrin β5 impacts spheroid viability

As a next step, it was important to evaluate β5 integrin function in anchorage-independent conditions since this is a critical step in EOC metastasis. After siRNA-mediated knockdown of ITGβ5, spheroid viability analysis was performed by Trypan blue exclusion cell counting using a TC10 automated cell counter. The loss of integrin β5 does not result in any overt change in spheroid phenotype compared to control (Figure 3.5A). Although there is no significant change in spheroid viability from integrin β5 knockdown in normal FT190 cells at 24 hours, there is a significant loss of viable cells by 72 hours in suspension culture (Figure 3.5B, 3.5C). OVCAR8 cells exhibited a decrease in spheroid viability at 24 hours from the loss of integrin β5, however, this decrease is not sustained at 72 hours (Figure 3.5B, 3.5C). Similarly, ES-2 cells have a significant decrease in spheroid viability at 24 hours and continue to have a decreased trend in the number of viable cells at 72 hours (Figure 3.5B, 3.5C). Interestingly, there is a significant increase in the number of viable HEYA8 cells at 72 hours and TOV21G cells in spheroid culture at both 24 and 72 hours (Figure 3.5B, 3.5C). CellTiter-Glo Luminescence cell viability assay was used as an additional method to assess spheroid cell viability based upon ATP as an indicator of metabolically-active viable cells. As indicated by previous spheroid viability results, ITGβ5 knockdown results in a significant increase in the average luminescence in TOV21G cells (Figure 3.5D). However, all other cells lines showed no difference in spheroid cell viability based on CellTiter-Glo assay (Figure 3.5D).
Figure 3.3: Transient knockdown of integrin β5 leads to a decrease in cell viability of EOC cells in adherent culture

(A) Immunoblot analysis validating ITGβ5 knockdown in FT190, OVCAR8, HEYA8 TOV21G, ES-2. Tubulin is used as a loading control. (B) Flow cytometry analysis of the ITGβ5 knockdown in FT190, OVCAR8, TOV21G and ES-2 cells transfected with control siNT or siITGβ5 using αvβ5-antibody. (C) The average αvβ5 fluorescence of cells was calculated. The results are presented as the mean±SD. Analysis was performed using two-tailed student’s t-test (****, P < 0.0001; ***, P < 0.001) (n=3). (D) Cell viability for FT190, OVCAR8, HEYA8, TOV21G and ES-2 siNT and siITGβ5. Viable cell number was determined by Trypan blue exclusion cell counting at 24 and (E) 72 hours in adherent culture. Data is presented as viable cell number normalized to their respective controls. Analysis was performed using two-tailed student’s t-test (NS= not significant;*, P < 0.05; **, P < 0.01; ***, P < 0.001).
Figure 3.4: Integrin β5 knockdown decreases cell adhesion in EOC without altering cell proliferation
(A) Images of FT190, OVCAR8, HEYA8, TOV21G, ES-2 siNT control and siITGβ5 cultured for 24 hours in adherent culture plates. Scale bars 200 μm. Images were captured using Leica light microscope (n=3). (B) Single cell adhesion was quantified with Trypan blue cell counting for FT190, OVCAR8, HEYA8, TOV21G and ES-2 siITGβ5 and matched controls. Analysis was performed using two-tailed student’s t-test (*, *P < 0.05) (n=3). (C) Doubling time for FT190, OVCAR8, HEYA8, TOV21G and ES-2 cells transfected with siNT or siITGβ5. Incucyte Zoom Imaging system measured percent confluence over 7 days. Graphpad PRISM was used to generate growth curves and non-linear regression analysis calculated doubling time. (NS= not significant). (n=3). Graphpad PRISM was used to generate growth curves and non-linear regression analysis calculated doubling time. (NS= not significant). (n=3).
Figure 3.5: Transient knockdown of integrin β5 impacts spheroid viability

(A) Images of FT190, OVCAR8, HEYA8, TOV21G and ES-2 siNT and siITGβ5 in spheroid culture for 24 hours. Scale bars 500 μm. Images were captured using Leica light microscope (n=3) (B) Spheroid viability for FT190, OVCAR8, HEYA8, TOV21G and ES-2 siNT and siITGβ5. Viable cell number was determined by Trypan blue exclusion cell counting at 24 hours and (C) 72 hours. Data is presented as viable cell number normalized to their respective controls. Analysis was performed using two-tailed student’s t-test (NS= not significant; *, P < 0.05; **, P < 0.01; *** P < 0.001) (n=3). (D) Spheroid viability for FT190, OVCAR8, HEYA8, TOV21G and ES-2 siNT and siITGβ5. Average luminescence was determined by CellTiter-Glo assay 24 and 72 hours after transfection. Data is presented as absolute viable cell number. The results are presented as the mean±SD. (*, P < 0.05) (n=3)
3.6. Loss of integrin β₅ decreases spheroid reattachment in OVCAR8 spheroids and impacts subsequent cell spreading in multiple EOC cells

To evaluate the role of integrin β₅ in a model of secondary metastasis, cell lines that were transfected with either control or siITGβ₅ were seeded to form spheroids. Spheroids were collected at 24 and 72 hours to perform re-attachment to tissue culture-treated plates. The loss of integrin β₅ results in no significant difference in relative spheroid reattachment for FT190, HEYA8, TOV21G, and ES-2 spheroids at both 24 (Figure 3.6A, 3.6B) and 72 hours (Figure 3.6A, 3.6C). Although there is a decreasing trend of spheroid reattachment in TOV21G and ES-22, there is an increasing trend in spheroid reattachment in HEYA8 spheroids at both time points (Figure 3.6B, 3.6C). Surprisingly, OVCAR8 cells show a dramatic decrease in the ability of spheroids to reattach at both 24 and 72 hours due to ITGβ₅ knockdown (Figure 3.6A, 3.6B, 3.6C).

In addition to quantifying total spheroid reattachment within an entire well, we investigated the change in spheroid area for individual reattached spheroids. Dispersion area was calculated by subtracting the spheroid core area from the total spheroid area as determined using Image J FIJI segmentation plugin. However, FT190 and HEYA8 spheroids do not have a measurable spheroid core so it was not possible to calculate dispersion area using this method (Figure 3.6D). For these two cell lines, the quantification for single spheroids is shown as total reattached spheroid area. The FT190 reattached spheroids show no difference in spheroid area at 24 hours (Figure 3.6D, 3.6E), but there is a significant decrease in spheroid area at 72 hours in the ITGβ₅ knockdown condition (Figure 3.6D, 3.6F). At 24 hours, both OVCAR8 and ES-2 show a significantly decreased ability of cells to disperse from the spheroid core once attached to tissue culture-treated substratum (Figure 3.6D, 3.6E). Although OVCAR8 spheroids are larger in size after 72 hours of spheroid culture, the altered ability of cells to disperse is still evident due to the loss of integrin β₅ (Figure 3.6D, 3.6F). Although there was no significant difference in dispersion area for TOV21G reattached spheroids at both time points and for ES-2 at 72 hours, there was a change in spheroid morphology (Figure 3.6D, 3.6E, 3.6F). Interestingly, HEYA8 reattached spheroids have a significantly larger spheroid area when integrin β₅ is knocked down at both 24 and 72 hours (Figure 3.6D, 3.6E, 3.6F). The loss of integrin β₅ within the HEYA8 spheroids, exhibited a similar
change in cell phenotype as seen with TOV21G and ES-2 in addition to an intensive spheroid core (Figure 3.6D).

### 3.7. Decrease in spheroid reattachment and cell spreading in OVCAR8 spheroids associated with integrin β5 and vitronectin interaction

A critical step in secondary tumor formation is the integrin-mediated binding of EOC cells to mesothelial cells and the underlying ECM. We assessed the interaction of integrin β5, and its associated αv integrin subunit capable of binding with RGD associated ECM ligands fibronectin and vitronectin [15], while bovine serum albumin (BSA) was used as a negative control. Similar to previous spheroid reattachment assay, spheroids for each cell line were cultured in ultra-low attachment plates for 24 hours transferred spheroids to ECM-coated adherent culture plates for 24 hours. The results indicate that knocking down ITGβ5 does not impact spheroid reattachment to either the negative control or fibronectin for FT190 (Figure 3.6A), OVCAR8 (Figure 3.6B), HEYA8 (Figure 3.6C) and TOV21G (Figure 3.6D). The results did indicate a significant decrease in the ability of OVCAR8 spheroids to reattach in vitronectin-coated wells when ITGβ5 is knocked down (Figure 3.6B). However, this change due to ITGβ5 knockdown in spheroid reattachment with vitronectin was not evident in FT190 (Figure 3.6A), HEYA8 (Figure 3.6C) and TOV21G (Figure 3.6D). These results indicate that integrin β5 may play a role in spheroid reattachment when interacting with vitronectin in OVCAR8 cells.

To investigate the change in subsequent cell spreading after spheroid reattachment seen in previous spheroid reattachment experiment, we assessed the interaction of integrin β5 with ECM ligands fibronectin, vitronectin and BSA. There was no significant difference in subsequent cell spreading in FT190 (Figure 3.8A), HEYA8 (Figure 3.8C), TOV21G (Figure 3.8D) spheroids when adhering to BSA, fibronectin and vitronectin coated wells. Interestingly, loss of integrin β5 resulted in more dense reattached HEYA8 spheroids under both fibronectin and vitronectin conditions (Figure 3.8C). The results did indicate a dramatic decrease in the ability of OVCAR8 cells to spread from the reattached spheroid core when the wells were coated with vitronectin (Figure 3.8B).
Figure 3.6: Loss of integrin β5 decreases spheroid reattachment in OVCAR8 and impacts subsequent cell spreading in multiple EOC cells

(A) Spheroid reattachment Hema-3 stained Images of FT190, OVCAR8, HEYA8, TOV21G and ES-2 siNT and siITGβ5 in spheroid culture for 24 and 72 hours. Images were captured using Axio zoom microscope 9.8x (n=3) (B) Relative spheroid reattachment total area quantification for FT190, OVCAR8, HEYA8, TOV21G and ES-2 siNT and siITGβ5 at 24 hours. Total area was determined by Image J Segmentation Plugin analysis. Data is presented as total stained area and normalized to their respective controls. Analysis was performed using two-tailed student’s t-test (NS= not significant;*, P < 0.05). The results are presented as the mean±SD. (n=3). (C) Relative spheroid reattachment total area quantification for FT190, OVCAR8, HEYA8, TOV21G and ES-2 siNT and siITGβ5 at 72 hours. Total area was determined by Image J Segmentation Plugin analysis. Data is presented as total stained area and normalized to their respective controls. Analysis was performed using two-tailed student’s t-test (NS= not significant;**, P < 0.01). The results are presented as the mean±SD. (n=3). (D) High magnification spheroid reattachment Hema-3 stained Images of FT190, OVCAR8, HEYA8, TOV21G and ES-2 siNT and siITGβ5 in spheroid culture for 24 and 72 hours. Scale bar FT190 and OVCAR8 200 μm. Scale bar HEYA8, TOV21G and ES-2 500 μm. Images were captured using Leica light microscope (n=3). (E) Relative spheroid dispersion area quantification for FT190, OVCAR8, HEYA8, TOV21G and ES-2 siNT and siITGβ5 at 24 hours. FT190 and HEYA8 spheroid area was determined by quantifying total area of the reattached spheroid. Dispersion area was determined by subtracting spheroid core area from the total area of the spheroid using Image J Segmentation Plugin analysis. Data is presented normalized to their respective controls. Analysis was performed using two-tailed student’s t-test (NS= not significant;*, P < 0.05; ***, P < 0.01). The results are presented as the mean±SD. (n=3). (F) Relative spheroid dispersion area quantification for FT190, OVCAR8, HEYA8, TOV21G and ES-2 siNT and siITGβ5 at 72 hours. FT190 and HEYA8 spheroid area was determined by quantifying total area of the reattached spheroid. Dispersion area was determined by subtracting spheroid core area from the total area of the spheroid using Image J Segmentation Plugin analysis. Data is presented normalized to their respective controls. Analysis was performed using two-tailed student’s t-test (NS= not significant;*, P < 0.05). The results are presented as the mean±SD. (n=3).
Figure 3.7: Decrease in spheroid reattachment in OVCAR8 spheroids associated with integrin β5 and vitronectin interaction

(A) Spheroid reattachment Hema-3 stained Images of FT190 siNT and siITGβ5 using either Bovine Serum Albumin (BSA), fibronectin or vitronectin coated adherent tissue culture plates at 24 hours. Images were captured using Axio zoom microscope at 6.6x. Relative spheroid reattachment total area quantification for FT190 siNT and siITGβ5 at 24 hours. Total area was determined by Image J Segmentation Plugin analysis. Data is presented as total stained area and normalized to their respective controls. Analysis was performed using two-tailed student’s t-test. The results are presented as the mean±SD. (n=3).

(B) Spheroid reattachment Hema-3 stained Images of OVCAR8 siNT and siITGβ5 using either BSA, fibronectin or vitronectin coated adherent tissue culture plates at 24 hours. Images were captured using Axio zoom microscope at 6.6x. Relative spheroid reattachment total area quantification for OVCAR8 siNT and siITGβ5 at 24 hours. Total area was determined by Image J Segmentation Plugin analysis. Data is presented as total stained area and normalized to their respective controls. Analysis was performed using two-tailed student’s t-test (*, P < 0.05). The results are presented as the mean±SD. (n=3).

(C) Spheroid reattachment Hema-3 stained Images of HEYA8 siNT and siITGβ5 using either BSA, fibronectin or vitronectin coated adherent tissue culture plates at 24 hours. Images were captured using Axio zoom microscope at 6.6x. Relative spheroid reattachment total area quantification for HEYA8 siNT and siITGβ5 at 24 hours. Total area was determined by Image J Segmentation Plugin analysis. Data is presented as total stained area and normalized to their respective controls. Analysis was performed using two-tailed student’s t-test. The results are presented as the mean±SD. (n=3).

(D) Spheroid reattachment Hema-3 stained Images of TOV21G siNT and siITGβ5 using either BSA, fibronectin or vitronectin coated adherent tissue culture plates at 24 hours. Images were captured using Axio zoom microscope at 6.6x. Relative spheroid reattachment total area quantification for HEYA8 siNT and siITGβ5 at 24 hours. Total area was determined by Image J Segmentation Plugin analysis. Data is presented as total stained area and normalized to their respective controls. Analysis was performed using two-tailed student’s t-test. The results are presented as the mean±SD. (n=3).
Figure 3.8: Decrease in subsequent cell spreading after reattachment in OVCAR8 spheroids is associated with integrin β5 and vitronectin interaction

(A) High magnification spheroid reattachment Hema-3 stained Images of FT190 siNT and siITGβ5 on BSA, fibronectin, and vitronectin coated plates for 24 hours. Scale bar 500 μm. Images were captured using Leica light microscope (n=3). Absolute spheroid area quantification for FT190 siNT and siITGβ5 at 24 hours. Total area was determined by Image J Segmentation Plugin analysis. Data is presented as total reattached spheroid area. Analysis was performed using two-tailed student’s t-test. The results are presented as the mean±SD. (n=3).

(B) High magnification spheroid reattachment Hema-3 stained Images of FT190 siNT and siITGβ5 on BSA, fibronectin, and vitronectin coated plates for 24 hours. Scale bar 500 μm. Images were captured using Leica light microscope (n=3). Absolute spheroid area quantification for FT190 siNT and siITGβ5 at 24 hours. Total area was determined by Image J Segmentation Plugin analysis. Data is presented as total reattached spheroid area. Analysis was performed using two-tailed student’s t-test. The results are presented as the mean±SD. (n=3).

(C) Spheroid reattachment Hema-3 stained Images of HEYA8 siNT and siITGβ5 using either BSA, fibronectin or vitronectin coated adherent tissue culture plates at 24 hours. Scale bar 500 μm. Images were captured using Leica light microscope (n=3). Relative spheroid reattachment total area quantification for HEYA8 siNT and siITGβ5 at 24 hours. Total area was determined by Image J Segmentation Plugin analysis. Data is presented as total stained area and normalized to their respective controls. Analysis was performed using two-tailed student’s t-test. The results are presented as the mean±SD. (n=3).

(D) Spheroid reattachment Hema-3 stained Images of TOV21G siNT and siITGβ5 using either BSA, fibronectin or vitronectin coated adherent tissue culture plates at 24 hours. Scale bar 500 μm. Images were captured using Leica light microscope (n=3). Relative spheroid reattachment total area quantification for HEYA8 siNT and siITGβ5 at 24 hours. Total area was determined by Image J Segmentation Plugin analysis. Data is presented as total reattached spheroid area and normalized to their respective controls. Analysis was performed using two-tailed student’s t-test. The results are presented as the mean±SD. (n=3).
3.8. References


4. Discussion

4.1. Summary of findings

My thesis provides insight into the previously unknown role of integrin β5 in EOC. We have shown that integrin β5 mRNA expression is detectable across serous ovarian tumours and cell lines. The protein expression of integrin β5 varies across numerous well-established cell lines and patient-ascites derived cell lines. Previously, Kaplan-Meier analysis has shown that elevated integrin β5 levels in ovarian cancer are significantly associated with decreased patient survival and increased immunohistochemistry staining of integrin β5 in advanced stages II-IV compared to normal ovary tissue and stage I serous tumors [91]. My transient knockdown data indicates that there is a decrease in cell viability within adherent culture across multiple EOC cell lines. Alongside the change in viable cell number, there was a significant decrease in the ability of cells to adhere to tissue culture substratum. The results indicate that there is no change in proliferation rate for all cell lines evident through the doubling time analysis. When studying spheroids derived from EOC cell lines, we show that there is no visible change in spheroid morphology when integrin β5 is knocked down. Although our results indicate a decrease in spheroid viability in three of the five cell lines, there is also a significant increase in the number of viable cells in the other two cell lines. To further explore the role of integrin β5 in EOC, we evaluated the role of integrin β5 in spheroid reattachment. The results indicate that the ability of OVCAR8 spheroids to reattach and cell dispersion is significantly decreased. Alternatively, there is an increase in the dispersion area of HEYA8 spheroids although there was no change in the ability of these spheroids to reattach. Perhaps spheroid viability contributes to the ability of
spheroids to reattach and then disperse. Spheroid viability data suggests that knockdown of integrin β5 leads to decreased OVCAR8 spheroid viability whereas, HEYA8 spheroids have increased viability. Lastly, we investigated the role of integrin β5 in integrin-mediated EOC spheroid reattachment to various ECM ligands. The results demonstrate that the decrease in OVCAR8 spheroid reattachment and subsequent cell spreading is associated with integrin β5—vitronectin interaction.

4.2. **Integrin β5 knockdown decreases cell adhesion in EOC**

Cell adhesion interactions are involved in numerous physiological processes such as wound healing and embryonic development as well as the progression of diseases such as cancer [115]. One of the best characterized cell adhesion receptors are integrins, which can play a critical role in cell-cell or cell-matrix interactions [3, 4]. Integrins can assemble various forms of cell-matrix adhesion such as clustering in focal adhesions and the formation of a mechanical link between ECM and intracellular actin bundles [3, 4]. The physical interaction link between cells and ECM proteins are the reason integrins are considered mechanosensing receptors [118]. The first step of mechanosensing is the conformation change of integrins by moving from a low to high affinity conformation through outside-in or inside-out signaling to form integrin clusters on the membrane [118]. Previously, it has been demonstrated there is an enrichment of integrin β5 to specific adhesion structures during interphase when analyzing cell cycle stages [119]. In our study, we showed that the transient knockdown of integrin β5 leads to a significant decrease in the ability of FT190, OVCAR8 and TOV21G cells to attach to tissue substratum. These results may reflect on the change in signal transductions from the loss of integrin β5 and disruption of cell adhesion interactions.
One aspect of cell detachment is facilitated by integrin-mediated upregulation of MMPs and activation of EMT [36]. For instance, the clustering of α2β1 and α3β1 enables the induction of MMP9 that leads to E-cadherin cleavage and cell-cell adhesion loosening through an Src kinase-dependent manner [54]. Increased MMP9 expression in ovarian tumor tissues and ascites has been associated with disease reoccurrence and poor patient survival [120]. Src family kinases are involved in integrin-mediated signal transmission from the extracellular environment via FAK activation [121] or through β integrin cytoplasmic tail that can induce Src activation [122]. Current data has shown that inhibition of Src activity can block integrin-induced E-cadherin ovarian cancer cell dissociation [54]. Furthermore, the loss of cell adhesion to the substratum has been defined as anoikis, a form of cell death [123]. However, the use of an αv integrin blocking antibody was not associated with cell death despite the alteration of cell adhesion [45]. The inhibition of αv integrins resulted in detachment of IGROV1 cells from the substratum and altered SKOV-3 cell spreading [45]. There was also a decrease in cell growth and cell cycle progression from the αv integrin blockade [45]. This was associated with an inhibition of ILK activity and subsequent inhibition of PKB/Akt phosphorylation on serine-473 and upregulation of p27Kip1 [45]. Interestingly, our results differed regarding cell growth since we have shown that there is no significant change in doubling time across all five cell lines. This difference could be attributed to the fact that Cruet-Hennequart et al. measured proliferation by counting cells after a 48 hour treatment with αv integrin blocking antibody [45] whereas we calculated doubling time by measuring percent confluency over a 7 day period post transfection. Furthermore, our study focuses on knocking down only one of the αv integrins and so perhaps the loss of multiple αv integrins leads to an additive effect and the loss of only integrin β5 could be compensated by other αv integrins. Although there was a similar phenotype of cell detachment seen from the loss
of integrin $\beta_5$ in OVCAR8 in adherent culture, this phenotype was not further investigated. Therefore, it may be worth further exploration to evaluate the altered cell adhesion interactions.

4.3. **Loss of Integrin $\beta_5$ decreases spheroid reattachment in OVCAR8 spheroid and impacts subsequent cell spreading in multiple EOC cells**

EOC metastasis occurs through multicellular disaggregates, known as spheroids, which exfoliate from the primary tumor and can reattach at another location within the peritoneum or omentum to form a secondary tumor [5]. This form of metastasis suggests that cell-cell and cell-matrix mechanisms regulate EOC progression and therefore it is critical to understand the interactions leading to spheroid reattachment and invasion of the mesothelium [5]. Our results indicate that the transient knockdown of integrin $\beta_5$ leads to a dramatic decrease in spheroid reattachment and subsequent spheroid disaggregation on the cell culture plate substratum. Although, there was no significant change in spheroid reattachment among the other cell lines, there was also a decrease in single spheroid reattachment area with the FT190 at 72 hours. However, there was a significant increase in the spheroid dispersion area for the HEYA8 spheroids when integrin $\beta_5$ was knocked down. One aspect of the decrease in spheroid reattachment and subsequent spheroid reattachment could be attributed to the decrease in spheroid viability due to loss of integrin $\beta_5$ for the OVCAR8 and FT190 spheroids. Interestingly, HEYA8 spheroids have shown an increase in spheroid viability and spheroid disaggregation post reattachment. These results could suggest that changes in spheroid viability may contribute to spheroid reattachment.

Furthermore, the previous literature highlights the importance of EMT and change in the expression of various integrins could lead to changes in spheroid reattachment. For instance, the loss of E-cadherin leads to transcriptional upregulation of fibronectin and $\alpha_5\beta_1$ integrin, which is
essential when spheroids initiate adhesion at a secondary site [53]. Interestingly, Bianchi et al. have demonstrated that the β-subunits associated with α_v integrins are upregulated during TGF-β induced EMT in breast carcinoma [58]. Specifically, knockdown of integrin β5 blocks the EMT response to TGF-β which impairs the assembly of tight junctions and formation of cell-matrix adhesion structures in mouse and human mammary epithelial cell lines [58]. This suggests that integrin β5 cell-matrix adhesion interactions play a role in EMT process in response to TGF-β and the tumorigenic potential of carcinoma cells [58]. We have previously established that TGF-β activity is induced during ascites-derived EOC spheroid formation indicated by the upregulation of Snai1, Twist1, Twist2 and Zeb2 mesenchymal markers [57]. When spheroids were treated with either TGF-β receptor 1 inhibitor or DMSO control, there was reduced cell-cell cohesion within spheroids and a decrease in spheroid dispersion area [57]. The enhanced epithelial phenotype of dispersing cells treated with TGF-β inhibitor compared to the control may have contributed to the decrease in cell motility after reattachment. Similar to breast carcinoma [58], it may be possible that the regulation of integrin β5 expression and TGF-β induced EMT in EOC may impact spheroid reattachment and subsequent cell spreading. Since our previous work has shown the role of TGF-β activity in EOC spheroid formation and reattachment, it is important that future studies focus on elucidating a possible association of integrin β5 and TGF-β induced EMT.

4.4. Decrease in spheroid reattachment and disaggregation in OVCAR8 spheroids associated with Integrin β5 and vitronectin interaction

A critical step in secondary tumor formation is the integrin-mediated binding of EOC cells to the underlying ECM of the mesothelial layer. Many studies have shown that integrin β1 is a critical beta subunit since it can pair with a variety of alpha subunits and participate in EOC
cell attachment and migration on ECM substrata relevant to peritoneal metastasis [5]. This association has been seen in gastric cancer cells as well, where peritoneal invasion was inhibited by blocking integrin α2β1 association with collagen I and discouraging cancer cell attachment [124]. Our results indicate that knocking down integrin β5 results in a significant decrease in the ability of OVCAR8 spheroids to reattach and disaggregate on vitronectin-coated substrata. However, there was no significant difference in OVCAR8 spheroid reattachment to fibronectin or BSA. Although this potential role of integrin β5 has not been seen in EOC previously, the inhibition of other integrins has been associated with altered spheroid disaggregation. The inhibition of collagen-associated integrin α2β1 lead to attenuated spheroid disaggregation on artificial ECM [8,19, 20]. Burleson et al. have also shown that integrin β1 partially mediates adhesion of EOC spheroids to ECM and plays a more significant role in spheroid disaggregation [61]. When evaluating spheroid disaggregation on ECM-coated surfaces, the addition of an inhibitory antibody against integrin β1, eliminated OVCAR5 spheroid disaggregation on laminin, fibronectin and type-IV collagen to an extent and a 50% reduction on collagen I [61]. Although our results also indicated a significant decrease in the ability of OVCAR8 spheroids to reattach to a vitronectin-coated surface, integrin β1 blockade did not prevent initial spheroid reattachment [61].

Interestingly, the loss of integrin β5 did not prevent spheroid reattachment or disaggregation in the other EOC cell lines. It is known that integrins can play different roles in cell fate decisions. For instance, two gastric adenocarcinoma sublines differ in their response to anchorange-independent culture where one results in apoptosis and the other results in cell cycle arrest [125]. Our results indicate that inhibiting the interaction between integrin β5 and vitronectin impacts spheroid reattachment and disaggregation in the OVCAR8 but does not seem
to affect the other cell lines. Perhaps this can be attributed to the varying expression of other $\alpha_v$ integrins across these cell lines. For instance, it may be possible that another $\alpha_v$ integrin capable of binding with vitronectin is compensating for the loss of integrin $\beta_5$ and is able to engage in signaling pathways allowing for continued spheroid reattachment and subsequent cell spreading. Kligys et al. have shown that $\alpha_6\beta_4$ is a master regulator of transcription and translation of other integrin subunits and the loss of this heterodimer can decrease the expression of $\alpha_2$ and $\alpha_3$ integrin subunits [126]. They provide evidence that the $\alpha_6\beta_4$ integrin-dependent signaling via phosphorylation of 4EBP1 and activation of PI3K regulates the translation of other integrin subunits such as $\alpha_3$ [126]. While $\alpha_v$ integrins also interact with vitronectin as an ECM ligand, it does not imply that the cell-matrix adhesions will lead similar changes in cell motility. The difference in cell migration between two ECM-binding integrins can involve different signaling and/or adaptor proteins interacting with differing amino acid sequences on the cytoplasmic tails of integrin subunits [127]. In this study, we have knocked down integrin $\beta_5$, however other beta subunits that bind with $\alpha_v$ integrin can differ in their cytoplasmic tail and recruit varying adaptor proteins forming a different signaling platform [127]. Overall, altering the expression of integrins and their composition of cell-matrix adhesion complexes may be a driving force in cancer progression processes.

4.5. Limitations of current study

My work helped to begin the process of uncovering the function of integrin $\beta_5$ in EOC, however there are some aspects of the study that have limitations. This can be explored and addressed further through additional experimentation.
Firstly, the single cell adhesion assays in adherent culture showed that some cell lines, the
loss of integrin $\beta_5$ led to the formation of spontaneous cell clusters that were suspended in the
media. Previous literature has shown that these cell clusters can form by budding directly from a
monolayer where budding was associated with vertical growth, continued cell-cell interactions
and eventual release of the cell cluster [34]. Pease et al. reveal that the cell lines capable of
forming these cell clusters in adherent culture showed a lack of E-cadherin at cell-cell borders
and the presence of vimentin filaments throughout the cytoplasm through immunofluorescence,
indicating the cells have undergone EMT [34]. To evaluate the altered cell adhesion properties
between the cells in monolayer and the cell clusters in the media, it may be helpful to collect the
supernatant with the cell clusters separate monolayer to perform microarray analysis. This may
provide insight into changes in gene expression from the transient knockdown of integrin $\beta_5$
between the cell clusters and monolayer. Furthermore, comparing the supernatant lysates with
the monolayer using western blot analysis to probe for EMT associated proteins may provide
advantageous in further evaluating the role of integrin $\beta_5$ and cell adhesion interactions.

In addition, the spheroid reattachment experiments provided valuable insight into the
function of integrin $\beta_5$ in secondary metastasis and the interaction with ECM proteins. Our study
shows that the interaction between integrin $\beta_5$ and vitronectin is important for OVCAR8
spheroid reattachment and subsequent cell spreading. However, there are multiple aspects of
secondary metastasis that ultimately reinforce locally invasive behaviour of EOC such as, ECM
stiffness that increases integrin signaling and subsequent activation of associated pathways [128].
A study by McKenzie et al. investigated the tumor microenvironment that regulates EOC
morphology, migration and spheroid disaggregation using polymer hydrogels with elastic
properties that mimic those of the peritoneum [128]. The peritoneum represents a major target
for EOC dissemination and polyacrylamide hydrogels that were fabricated with physiologically appropriate ECM stiffness serve as experimentally relevant adhesive substrates [128]. These gels can then be coated with different ECM proteins and can help to evaluate EOC cell size, actin cytoskeletal organization and focal adhesion morphology through immunofluorescence [128]. Moreover, this method provides a physiologically relevant model of the peritoneum to evaluate spheroid reattachment and disaggregation. Alternatively, Kenney et al. have established a 3D model of the key components of the omental microenvironment, mesothelial cells, fibroblasts and ECM, to study ovarian cancer cell adhesion and invasion [129]. They provide evidence that omental mesothelial cells inhibit, while omental fibroblasts and underlying ECM enhance the attachment and invasion of EOC cells [129]. The use of this model provides the ability to study EOC spheroid attachment and disaggregation in the context of the microenvironment which is critical in EOC metastasis.

Furthermore, it is critical to understand integrin crosstalk and the diverse number of compensatory mechanisms involved. Our work focuses on investigating the function of integrin β5 and we have assessed this through siRNA-mediated knockdown. However, this integrin subunit is only one of another 7 beta subunits and 18 alpha subunits. Integrins possess an innate characteristic where the change in the expression of integrin subunits or the activation of certain integrins can interfere with the expression or activation of another [130]. For instance, the expression of integrin α3 decreases the activation of αv, while inhibiting integrin α3 leads to the activation of αvβ3 expression in several cancer cell types [131]. Furthermore, in the melanoma cell line MDA-MB-435S which expresses integrins β3 and β5, the knockdown of β3 or β5, upregulates αvβ5 and αvβ3 respectively [132]. Therefore, western blot analysis to explore whether
other αν integrins are engaging in compensation mechanisms when integrin β5 is knocked down may provide insight into the different phenotypes observed across the cell lines.

Lastly, spheroid formation and subsequent reattachment is an essential step in EOC metastatic cascade. To mimic spheroid formation and subsequent reattachment in this study, a 3D spheroid culture model was used to form spheroids in ULA. These spheroids were then reattached in adherent culture plates to mimic secondary tumor formation. When completing these experiments, the spheroids were reattached in media contained FBS. For in vitro cell expansion, ECM components of FBS as well as other materials of animal origin are required for cell culture [31]. The use of 10% FBS when completing the spheroid reattachment assays is a limitation of the experiment since the ECM components within the media can coat the plastic surface of the well and play a role on cell adhesion. In the future, the use of cell culture media without FBS in spheroid reattachment assays can address this restriction. This limitation was addressed going forward in the spheroid reattachment assays with ECM-coated wells where the spheroids were reattached in media with no FBS.

4.6. Overall Conclusions

My work has contributed to the growing literature evaluated the role of the integrin family in cancer progression. I have shown that integrin β5 may play a role in EOC and knockdown of this beta subunit decreases cell adhesion across multiple cell lines. Furthermore, the results indicate that the loss of integrin β5 has a dramatic impact on spheroid reattachment subsequent cell spreading in the OVCAR8 cell line. OVCAR8 is a HGSOC cell line that has rapid tumor formation ability and for intraperitoneal injections in a xenograft mouse model, 100% tumor take has been seen [32]. Compared to the other OVCAR cell lines, OVCAR8 is the
only one that can reliably form ascites in a xenograft mouse model within 90 days [32].

OVCAR8 also forms the largest intraperitoneal tumors (1004-1509 mg) by 27 days [32].

Functional characterization in vitro showed that OVCAR8 has the most mesenchymal phenotype compared to other HGSOC cell lines such as OVCAR5, OVSAHO, SNU119 and CAOV3 [32].

Although, we have gained new insights into the influence of integrin βs in the context of EOC, it is vital to continue further experimentation of the results prior to making final conclusions. My results elucidate that complexity of integrin-mediated cell adhesion interactions in EOC, since the phenotypes differ across the different cell lines evaluated. By uncovering the expression of other integrin heterodimers and possible integrin cross talk, future studies may provide insight into the diversity of integrin function among EOC tumors. Integrins are key regulators at various steps in the EOC metastatic cascade; therefore, if we can continue to elucidate potential integrin-mediating signaling mechanisms, it can potentially address the challenges of using integrin inhibitors as EOC therapeutics currently in clinical trials.
4.7. References


