MICROENVIRONMENTAL REGULATION OF OVARIAN CANCER DISSEMINATION VIA ACTIVATION OF THE WNT SIGNALING PATHWAY

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by
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The undersigned, appointed by the Dean of the Graduate School, have examined the dissertation entitled

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I have completed this work by the grace and mercy of God, to whom all praise is given.

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LIST OF ABBREVIATIONS

2D	Two-Dimensional
3D	Three-Dimensional
AJ	Adherens Junction
APC	Adenomatous Polyposis Coli
CK	Casein Kinase
DHAP	Dihydroxyl Acetone Phosphate
Dkk	Dickkopf
Dvl	Dishevelled
ECM	Extracellular Matrix
EMT	Epithelial-to-Mesenchymal Transition
EOC	Epithelial Ovarian Carcinoma
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
FAK	Focal Adhesion Kinase
FGF	Fibroblast Growth Factor
FN	Fibronectin
Fzd	Frizzled
GSK-3β	Glycogen Synthase Kinase – 3 Beta
IGF	Insulin-Like Growth Factor

IL	Interleukin
ILK	Integrin Linked Kinase
Lef	Lymphoid Enhancer Factor
LINC	Linker of Nucleoskeleton and Cytoskeleton
LPA	Lysophosphatidic Acid
LRP	Lipoprotein Receptor-Related Protein
MAG	Monoacylglycerol
MCA	Multicellular Aggregates
MET	Mesenchymal-to-Epithelial Transition
MIDAS	Metal Ion-Dependent Adhesion Site
MMP	Matrix Metalloprotease
MSC	Mechanosensitive Channel
NGFR	Nerve Growth Factor Receptor
OCAF	Ovarian Cancer Activating Factor
OSE	Ovarian Surface Epithelium
PC	Phosphatidylcholine
PCP	Planar Cell Polarity
PDGFR	Platelet-Derived Growth Factor Receptor
PECAM	Platelet/Endothelial Cell-Adhesion Molecule
PI3-K	Phosphatidylinositol 3-Kinase

PKC	Protein Kinase C
PLA	Phospholipase A
PLD	Phospholipase D
PPAR	Peroxisome Proliferator-Activated Receptor
PTEN	Phosphatase and Tensin Homolog
ROCK	Rho-Associated Kinase
SFRP	Secreted Frzzled-Protein Related Protein
Tcf	T-Cell Factor
TGF-β	Transforming Growth Factor - Beta
TJ	Tight Junction
uPA	Urinary Plasminogen Activator
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
WIF	Wnt Inhibitory Factor

1. INTRODUCTION AND BACKGROUND

I. INTRODUCTION TO OVARIAN CANCER

Statistics.

Epithelial ovarian carcinoma (EOC) is the leading cause of death from gynecologic malignancies. In 2011 there were an estimated 21,990 new cases of, and over 15,000 deaths from, epithelial ovarian carcinomas. Less than 30% of patients survive 5 years after developing distant metastases, compared with a 92% 5-year survival of patients with localized tumors [National Cancer Institute, 2011]. Greater than 80% of ovarian cancer patients will present with metastasizing disease, highlighting the need for elucidation of the mechanisms that underlie the ovarian tumor cell metastatic process.

Etiology of Epithelial Ovarian Carcinoma.

Although the vast majority of epithelial ovarian carcinomas present histologically as dedifferentiated epithelial-type cells and are though to arise from the normal ovarian surface epithelium (OSE), the etiology of EOC is poorly understood [DuBeau, 1999]. The normal ovarian epithelium is a single cell layer supported by a basement membrane comprised of dense collagenous connective tissue [Auersperg et al, 1994]. The OSE is derived from the mesoderm, and displays both epithelial and mesenchymal characteristics expressing both keratin and vimentin. In tissue culture, OSE cells express epithelial markers including keratin, laminin, and type IV collagen, as well as mesenchymal markers such as vimentin and interstitial collagen types I and III [Czernobilsky et al, 1985]. The OSE undergoes reversible transition to a fibroblastic phenotype during post-ovulatory repair of the epithelium [Auersperg et al, 1994]; this phenotypic plasticity suggests that OSE adapts to changes in the cellular microenvironment by transition between epithelial and mesenchymal phenotypes, a characteristic usually limited to immature, healing, or tumorigenic epithelia. Studies of ovarian tumor initiation have given rise to two major theories of ovarian tumor etiology. It is widely accepted that EOCs arise from either the single-cell layer of epithelium lining the ovary or from aberrations in structures within, such as clefts or inclusion cysts [Radisavljevic, 1977; Scully, 1995; Auersperg, 1998]. In this theory, abnormalities in post-ovulatory wound repair or remodeling secondary to pregnancy or aging may give rise to tumorigenic mutations. Alternatively, a second theory suggests atypical modifications in the secondary mullerian

system, adjacent to the ovary, are the source from where EOC arises [DuBeau, 1999].

Ovarian Tumor Metastasis.

Ovarian tumors are pathologically heterogeneous with distinct modes of pathogenesis characterized by specific genetic alterations and unique molecular signatures [Sheddan et al, 2005; Shih and Kurman, 2004; Schwartz et al, 2002]. These signatures allow classification of EOCs, loosely, into four histotypes: serous, endometrioid, mucinous, and clear cell. Clinically, tumors often involve the ovary and omentum, with multiple intraperitoneal metastases and accumulation of malignant ascites [Scully, 1998]. The metastatic cascade is initiated by exfoliation of single tumor cells and clusters of cells known as multicellular aggregates (MCAs) from the ovary surface [Burleson et al, 2004; Burleson et al, 2006]. This mesenchymal-to-epithelial (MET)-like transition is hallmarked by a gain of epithelial(E)- cadherin, compared with mesodermally-derived, neural(N)-cadherin expressing ovarian surface epithelium [Hudson et al, 2008; Wong et al, 1999; Sunfeldt et al, 1997; Patel, 2003]. Interestingly, this MET is transient, and further progression towards developing stable metastases appears to be dependent on a subsequent epithelial-to-mesenchymal transition (EMT) [Hudson et al., 2008].

Although the mechanisms are unclear, exfoliated tumor cells float and survive in

accumulating ascites in the peritoneal cavity, circumventing anoikis-related apoptosis signals [Naora and Montell, 2005]. These shed cells bind mesothelium throughout the peritoneal cavity, preferentially to peritoneal lymphatic beds in the greater omentum (known as milky spots) [Mutsaers et al, 2007; Sorenson et al, 2009; DiPaolo et al, 2005] thereby blocking reabsorption of inflammation-induced exudate, dysregulation of serum-ascites albumin gradient [Mactier et al, 1987], and accumulation of large volumes (500 ml up to 2 L) of ascites fluid [Rudlowski et al, 2006; Shen-Gunther and Mannel, 2002]. Distribution of exfoliated tumor cells is facilitated by peritoneal fluid, and formation of malignant ascites may further foster metastatic implantation. Ascites, a plasma exudate, is comprised of cellular components (ovarian cancer cells, lymphocytes), and acellular components including a variety of signaling factors including, but not limited to, lysophosphatidic acid (LPA), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), and inflammatory cells and signaling molecules [Puiffe et al., 2007; Cowden Dahl et al., 2007; Freedman et al, 2004; Lo et al, 2011]. The biologic components of the ascites are increasingly being shown to have major implications in the progression of ovarian cancer via diverse biochemical signaling pathways [Cowden Dahl et al., 2008; Said et al., 2007]. As ascites develops and stretches the peritoneum, vessel walls of the microvasculature are stretched, and prolonged stretching of the vessels are hypothesized to increase vessel size, resulting in hyperpermeation [Adair et al., 1990], further potentiating fluid accrual in the peritoneal cavity. Prolonged stretching of the peritoneum subsequently exposes the vessels to mechanical

modulation, facilitating extravasation of fibrinogen, which forms a thin stromal lining covering the peritoneal mesothelium [Nagy et al., 1995]. This finding is particularly significant because fibrinogen has been found to be upregulated in the peritoneum and stroma of patients with epithelial ovarian carcinoma, and is suggested to facilitate EOC metastasis [Wang et al., 2005; Olt et al., 1992; Ma et al., 2007].

Unlike most highly metastatic tumors, the majority of women with advanced intraperitoneal disease do not have metastases arising from vascular dissemination, suggesting a novel mechanism for metastasis is paramount in ovarian cancer progression [6, 168]. As the dissemination of ovarian cancer is largely contained within the peritoneal cavity, processes such as cell adhesion, migration and mesothelial/submesothelial invasion play a predominant role in ovarian cancer pathobiology.

II. LYSOPHOSPHATIDIC ACID

Lysophosphatidic Acid And Its Receptors.

Lysophosphatidic acid, LPA, is an intermediate in fatty acid metabolism and a phospholipid signaling molecule [Nagle et al, 2009]. It consists of a glycerol backbone and a hydroxyl group at the *sn-1* (**Fig. 1.1B**) or *sn-2* (**Fig. 1.1A**) position, a phosphate at *sn-3* position and a fatty acid chain at the *sn-2* (or *sn-1*) position. Most physiologically relevant LPA fatty acids are long chain and either saturated (C18:0, C16:0) or unsaturated (C18:1, C20:4) and are linked to the backbone by acyl- or alkyl-group [Pagès et al, 2001].

Ascites had been long shown to promote ovarian tumor growth. Ovarian cancer activating factor, OCAF, was identified as the primary functional component of ascites and is mostly composed of unsaturated fatty acids (multiple species of LPAs) [Xu et al, 1995a]. OCAF-derived LPAs are composed of a mixture of *sn-1* and *sn-2* LPAs; palmitoyl (16:0) LPA and oleoyl (18:1) are the most highly expressed LPA species in malignant ascites. Functional activity of OCAF is partially decreased by either phospholipase A_1 (PLA_1) or PLA_2 and completely

abrogated by $PLA_{1/2}$, demonstrating that ascites contains a *mixed composition of LPAs with acyl-linked fatty acids* [Xu et al, 1995b].

There are five proposed mechanisms by which LPA is produced, from intracellular and extracellular sources. LPA may be generated from: 1) glucose fatty acids or from 2) acyl dihydroxy acetone phosphate (acyl DHAP) by glycerol 3-phosphate (G3P). LPA can also be generated by 3) monoacylglycerol kinase (MAG-kinase) action on monoacylglycerol (MAG), as a precursor of phosphatidylinositol synthesis [Pagès et al, 2001]. 4) Phospholipase D (PLD) or 5) lysophospholipase D (lysoPLD) can generate acyl LPAs from phosphatidic acid (PA) or lysophosphatidylcholine (lysoPC), respectively [Pagès et al, 2001], and are the proposed mechanisms by which ovarian tumor cells produce LPA [Aoki, 2004].

The PLD biosynthetic process begins with PLD cleavage of phospholipids to form PA. *sn-1* PA is then further processed by PLA_1, while *sn-2* PA in processed by PLA_2. The lysoPLD pathway begins with processing of *sn-1* phospholipids by PLA_1 and *sn-2* phospholipids by PLA_2 to generate 1-acyl-2-lysoPLs and 2-acyl-2-lysoPLs, respectively. LysoPLD further processes these intermediates to form 1-acyl-2-lysoPAs (*sn-1* LPAs) and 2-acyl-1-lysoPAs (*sn-2* LPAs) [Aoki, 2004; Tokumura, 2002].

Ascites had been long shown to promote ovarian tumor growth [Mills et al, 1988; Mills et al, 1990]. Ovarian cancer activating factor, OCAF, was identified as the primary functional component of ascites and is mostly composed of unsaturated fatty acids (multiple species of LPAs). OCAF-derived LPAs are composed of a mixture of *sn-1* and *sn-2* LPAs; palmitoyl (16:0) LPA and oleoyl (18:1) are the most highly expressed LPA species in malignant ascites. Functional activity of OCAF is partially decreased by either PLA_1 or PLA_2 and completely abrogated by $PLA_{1/2}$, demonstrating that ascites contains a *mixed composition of LPAs with acyl-linked fatty acids* [Xu et al, 1995; Xiao et al, 2001].

LPA exacts its proliferative, migratory and invasive effects via signaling by activating its subfamily of G-protein coupled receptors, the LPA receptors. There are five known members of the receptor family, LPA_1 – LPA_5, which are encoded by distinct genes, *LPAR1-LPAR5* [Choi et al, 2010; Fukushima et al, 2001]. These ligand-receptor interactions modulate various signaling pathway proteins including Rho/ROCK (Rho-associated kinase), IP3/$Ca2^+$, PKC (protein kinase C), PI3K, Ras/MAPK (mitogen activated protein kinase) and cAMP [Choi et al, 2010; Fukushima et al, 2001]. Of these receptors, LPA_1 (Gα12/13), LPA_3 (Gαq/11) and LPA_4 (Gα12/13) receptor subtypes are expressed in the ovary, with LPA_4 being the most abundant. Receptor subtypes LPA_2 and LPA_3 are aberrantly overexpressed in several ovarian carcinoma cell lines [Choi et al, 2010; Fukushima et al, 2001; Fang et al, 2002]; this observation has been confirmed *in vivo* by detection of overexpressed LPA_2 and LPA_3 mRNA in human ovarian

tumor tissues, compared with normal and benign tissues [Fang et al, 2000; Fang et al, 2002]. LPA_1, a suggested pro-apoptotic and anti-proliferative LPA receptor is expressed in very low levels in many ovarian cancer cell lines; loss of LPA_1 expression has been suggested as a potential mediator of chemotherapeutic resistance in ovarian carcinomas [Furui et al, 1999]. It is interesting to note the existence of differential K_D values for LPA binding to each of its receptors. Binding affinity of LPA for LPA_2, LPA_3 and LPA_4 have been specifically measured (73.6 nM, 206 nM and 44.8 nM, respectively); however, binding affinity for LPA-LPA_1 has only been estimated (27 nM ± 3 nM) [Bandoh et al, 1999; Wang et al, 2001; Noguchi et al, 2003]. These data, taken together with evidence for the changes in receptor isoform expression discussed above, suggest that differential responses of EOC cells to LPA stimulation may be related to G protein subtype-dependent signaling but are independent of receptor responsiveness or sensitivity to LPA. To date, parameters of the ligand-receptor interaction between LPA and LPA_5 remain uncharacterized.

In addition to the five known LPA receptors, it has been recently proposed that LPA may interact with three orphan GPCRs (GPR87, P2Y5, and P2Y10) that are part of the P2Y superfamily [Murakami et al, 2008; Pasterneck et al, 2008; Shimomura et al, 2008; Tabata et al, 2007]. Of these three, P2Y5 is proposed as the next member of the LPA receptor family, putatively LPA_6, supported by evidence of low-affinity interactions with LPA in biochemical studies [Yanagida et al, 2009]. Interestingly, GPR87 and P2Y10 have both been reported to increase

intracellular Ca^{2+} mobilization [Murakami et al, 2008; Tabata et al, 2007]. LPA has also been shown to transactivate the growth factor receptors, epidermal growth factor receptor, EGFR [Symowicz et al, 2007; Zhao et al, 2006; Shah et al, 2005; Mori et al, 2006; Xu et al, 2007; Daaka, 2002], nerve growth factor receptor, NGFR [Moughal et al, 2006], and platelet-derived growth factor receptor, PDGFRβ [Wang et al, 2003], in a number of physiologic and pathologic conditions. Interestingly, LPA has also been shown to agonize a transcription factor that generally regulates genes related to energy metabolism, peroxisome proliferator-activated receptor γ (PPARγ). This binding affinity suggests PPARγ as the first identified intracellular receptor for LPA [McIntyre et al, 2003]. Signaling events downstream of LPA-induced transactivation of growth factors requires complex considerations of the role(s) of LPA in tumor progression.

LPA In The Ovarian Tumor Microenviornment.

LPA is highly expressed (up to 80 μM) in the ascites fluid and serum of patients with ovarian cancer, independent of disease staging [Westermann et al, 1998; Xu et al, 1995a, Xu et al, 1998; Xiao et al, 2001; Shen et al, 1998]. This overexpression is in stark contrast with other diseases such as hepatic disease, which is also marked by ascites accumulation and no significant increase in ascitic LPA expression [Mills et al, 1988]. Samples taken from women with ovarian cancer preoperatively and postoperatively demonstrate a significant decrease of plasma LPA expression in postoperative samples [Xu et al, 1998;

Sutphen et al, 2004]. Further, malignant effusions of women with ovarian cancer exhibit 5 times greater LPA species expression when compared with normal controls [Baker et al, 2002]. In addition to LPA produced by ovarian cancer cells [Shen et al, 1998; Eder et al, 2000], mesothelial cells, fibroblasts [Fukami et al, 1992; Moolenaar, 1994], adipocytes [Valet et al, 1998], inflammatory cells [Fourcade et al, 1995; Goetzl and An, 1998] and serum [Gerrard and Robinson, 1989; Eichholtz et al, 1993; Fourcade et al, 1995; Tokumura et al, 2002] can serve as sources of various species of LPA (stearoyl, arachadonoyl, and docosahexaenoyl) and contribute to LPA accumulation within the ascites microenvironment [Yu et al, 2008; Pagès et al 2001].

LPA In Physiology And Pathophysiology.

Owing in part to its diverse receptor subfamily, LPA mediates a vast number of cellular processes. LPA is known to potentiate cell proliferation, anti-apoptotic processes, cell differentiation, chemotaxis, migration and invasion [Moolenaar, 1999; Tokumura, 2002]. These responses to LPA are shown to have important implications in some physiologic and pathophysiologic processes. LPA stimulates platelet aggregation in response to stimuli including cellular damage, which, in addition to being part of the normal inflammatory response [Schumacher et al, 1979; Gerrard et al, 1979], has been implicated in the pathogenesis of athersclerosis [Rizza et al, 1999; Siess et al, 1999; Hayashi et al, 2001]. Lysophospholipase D (lysoPLD), which processes lysophosphatidylcholine

(lysoPC) into LPA, activity in humans is increased during pregnancy, and returns to normal levels within days following parturition [Tokumura et al, 2000]. While there is currently no data to suggest LPA plays a role in tumorigenesis, LPA signaling has been established as a potent mediator of pro-malignant behavior including colony scattering of epithelial cells and gain of mesenchymal phenotype [Shin et al, 2009; Jourquin et al, 2006].

In ovarian carcinoma, LPA mediates progression down the metastatic cascade in several ways. Previous work has shown that LPA is a potent mediator of both vascular endothelial growth factor receptor (VEGFR) [So et al, 2005; Hu et al, 2001] and epidermal growth factor receptor (EGFR) [Hudson et al, 2008; Hudson et al, 2009; Moss et al, 2009] signaling in ovarian cancer cells, which in turn positively regulate protease activity and Cox2 expression, respectively. LPA has also been shown to disrupt E-cadherin-based cell-cell adhesions and to facilitate invasion of ovarian carcinoma cells [Jourquin et al, 2006; Smicun et al, 2007; Symowicz et al, 2005; Fishman et al, 1997; Liu et al, in press]. Recent studies have shown that a panel of 39 LPA-induced genes presents a transcriptional profile, which correlates with decreased disease survival and decreased progression-free survival in ovarian cancer patients [Murph et al, 2009].

Gain of invasive phenotype is a hallmark of cancer, and mediated in large part by the activity of proteases [Hanahan and Weinberg, 2011]. LPA signaling leads to invasive behavior in ovarian carcinoma cells by increasing activity at the

(urokinase plasminogen activator) uPA promoter, mRNA expression levels, protein expression levels, secretion and enzymatic activity of uPA [Pustilnik et al, 1999; Estrella et al, 2007]. Additionally, LPA has been shown to regulate the activation of several matrix metalloproteinases (MMPs), namely membrane-type (MT)-MMP1, MMP-9, and MMP-2 [Fishman et al, 2001; Do et al, 2007; Symowicz et al, 2005]. Activation of proteases in ovarian carcinoma has been shown to promote E-cadherin ectodomain shedding, leading to disintegration of E-cadherin-based adherens junctions and promoting invasiveness [Gil et al, 2008; Liu et al, in press].

III. CELL ADHESION: INTEGRINS AND CADHERINS

The Integrin Family Of Receptors.

The integrins are a large family of transmembrane, adhesion molecules that couple the cellular cytoskeleton to extracellular matrix and facilitate cellular interaction with the microenvironment through “outside-in” and “inside-out” signaling mechanisms [Hynes, 2002]. Functionally, integrins are expressed as heterogenous dimers composed of one alpha and one beta subunit. Both the α and β subunits have large extracellular domains, and a short cytoplasmic domain (with the exception of the β4 subunit). The N-terminal region of the extracellular domain is composed is seven β-propeller domain repeats that fold into a seven-blade propeller domain; some with an inserted (I) domain between repeats 2 and 3 [Springer, 1997; Humphries, 2000]. The C-terminal region is comprised of a large stalk region containing 3 β-sandwich domains referred to as Thigh, Calf-1 and Calf-2 [Lu et al, 1998; Xiong et al, 2001]. The C-terminal extracellular region of the β subunit also exhibits a stalk region with four integrin-EGF (I-EGF) domains [Takagi et al, 2001; Xiong et al, 2001; Beglova et al, 2002]. The N-terminus begins with a plexins, semaphorins, and integrins (PSI) domain, named for its sequence homology to membrane proteins such as plexins, semaphorins

and the c-Met receptor [Bork et al, 1999]. This fifty amino acid domain is followed by hybrid domain, flanking a 240 residue I-like domain [Lee et al, 1995]. The tertiary structure of the I domain (α subunit) and the I-like domain (β subunit) are similar, and in quaternary structure the two share a large interface [Xiong et al, 2001]. Binding of ligand is dependent on the metal ion-dependent adhesion site (MIDAS), which contains a DXSXS binding motif and is found in both the I domain and the I-like domain [Lee et al, 1995; Xiong et al, 1999; Lu et al, 2001]. In the absence of the I domain in a heterodimer, I-like domain interacts directly with ligand; when I domain is present, I-like domain indirectly regulates ligand binding [Xiong et al, 2001]. Eighteen α and 8 β subunits comprise 24 distinct integrin heterodimers, which each have a specific, non-redundant function as receptors for diverse extracellular matrix ligands [Hynes, 1996; Plow, 2000; van der Flier, 2001]. Specificity is not only conferred through subunit expression, but also by receptor occupancy and receptor aggregation. Ligand-independent aggregation initiates clustering of integrin receptors and activation of downstream signaling through mechanisms such as tyrosine phosphorylation. Binding by monovalent ligand facilitates receptor redistribution to focal adhesion sites, while receptor occupancy by multivalent ligands synergistically couples ligand-independent and ligand-dependant signaling events [Miyamoto et al, 1995b; Connors et al, 2007].

The ‘switchblade’ model defines three major conformational states of the integrin heterodimer, which dictate activation status of the molecule: the inactive, low

affinity state, the active, high affinity state and the ligand-bound conformation [Beglova et al, 2002; Takagi et al, 2002; Xiong et al, 2002; Nishida et al, 2006; Askari et al, 2009]. In the low affinity or "bent" state, the cytoplasmic domain of the α subunit inhibits interaction between the β subunit and the cytoskeleton [Vinogradova et al, 2000]. Ligation of extracellular matrix (outside-in) or agonist-induced signaling (inside-out) initiates a conformational change to the high affinity or "extended" state, disrupting the inhibition of β subunit by α subunit [Hughes et al, 1996; Vinogradova et al, 2000]. Further, divalent cations such as Mn^{2+} may activate integrins by stabilizing or inducing the activated conformation [Diamond et al, 1994]. Differential signaling is activated based on matrix structure. Ligation of monovalent ligands mediate receptor re-localization to focal contacts, but little tyrosine phosphorylation-regulated signaling. Monovalent ligand-induced re-localization, integrin engagement of multivalent ligands, or interaction with a non-ligand aggregator leads to focal adhesion kinase (FAK)-dependent and cytoskeletal rearrangement (**Fig. 1.2**) [Miyamoto et al, 1995].

As cell-matrix adhesion molecules, integrins form complex focal adhesions at the cell-extracellular matrix interface that *integrate* the cytoskeleton and signaling during adhesion, migration, invasion, and response to extracellular forces [Miyamoto et al, 1995a; Jockush et al, 1995]. The hallmark characteristic of direct integrin signaling following adhesion is enhanced tyrosine phosphorylation [Kornberg et al, 1991]. Phosphorylation is mediated by the non-receptor tyrosine kinase, focal tyrosine kinase (FAK) [Schaller et al, 1992; Hanks et al, 1992]. FAK

auto-phosphorylation and recruitment of SH2-containing proteins (such as Src or Fyn) mediate phosphorylation of FAK at other sites [Cobb et al, 1994; Schaller et al 1994; Calalb et al, 1995], and allows for additional protein binding and activation of diverse pathways including Ras/ERK and PI3K/Akt [Vuori et al, 1996; Dolfi et al, 1998]. A major functional consequence of the recruitment of proteins to the integrin is cytoskeletal rearrangement in response to external stimuli. Activated FAK can recruit cytoskeletal proteins (like paxillin and talin, which in turn bind actin) and Rho GTPases to focal adhesions [Kiyokawa et al, 1998]. These events facilitate the induction of lamellipodia, filopodia and are necessary for integrin-mediated adhesion, spreading and migration [Schwartz and Shattil, 2000]. Integrins may also direct cell signaling through crosstalk with other pathways via five major mechanisms: 1) receptor transactivation, 2) receptor pathway modulation, 3) receptor coordination, 4) receptor compartmentalization, or 5) modulation of receptor expression [Miranti and Brugge, 2002].

β1 Integrin in Ovarian Carcinoma.

Disseminating ovarian tumor cells complete the metastatic cascade through successful adherence to and invasion through the mesothelial lining, and proliferation at the site of cell seeding [Wang et al, 2005]. As the mesothelial lining is inherently non-adhesive, several mechanisms of increased adhesivity have been proposed. The LP9 mesothelial cell line produces fibronectin, laminin,

and collagens type I, III and IV that lines the apical surface [Lessan et al, 1999; Lessan and Skubitz, 1998]. Mesothelial cells may also undergo tumor-induced retraction [Jayne et al, 1999; Hashimoto et al, 2003], shrinkage, or apoptosis [Heath et al, 2004] presenting the submesothelial matrix as an adhesive surface. Interestingly, it has also been proposed that tumor cell projections may physically 'push' mesothelial cells [Akedo et al 1986; Iwanicki et al, 2011], clearing a path for adhesion and invasion.

Normal ovary surface epithelial cells express α2, α3, α6, β1, and β4 integrin subunits; however, α6 and β4 subunits are downregulated in ovarian cancer cells [Skubitz 2002]. The α2β1 and α3β1 integrin heterodimers are ligands for collagen, suggesting they may play a role in tumor cell interaction with the collagen I/III-rich matrix that underlies the mesothelium [Moser et al, 1996; Mutsaers et al, 2007]. β1 integrin is expressed in ovarian carcinoma cell lines [Cannistra et al, 1995; Lessan et al, 1999], and mediates the adhesion of disseminating ovarian tumor cells both to the peritoneal mesothelium [Strobel et al, 1999] and submesothelial matrix [Cannistra et al, 1995; Lessan et al, 1999].

E-Cadherin Receptor.

Epithelial (E)-cadherin is a member of the superfamily of homotypic, calcium-dependent, cell-cell adhesion/recognition molecules known as the cadherins [Nollet et al, 2000]. The functional E-cadherin complex consists of β-catenin, α-

catenin and p120-catenin and couples cell-cell adhesion to the actin cytoskeleton [Aberle et al, 1994]. β-catenin binds the distal end of the cytoplasmic tail of E-cadherin and also binds α-catenin. In addition to binding β-catenin, α-catenin also binds actin directly. Finally, p120-catenin binds the membrane-proximal cytoplasmic tail of E-cadherin, completing the cadherin-catenin complex [Aberle et al, 1994; Daniel et al, 1995; Rimm et al, 1995; Kobielak et al, 2004; Drees et al, 2005]. There are three major types of cell-cell adhesions: tight junctions (TJs), adherens junctions (AJs) and desmosomes; functionally, E-cadherin is directly involved in formation of AJs and TJs [Gumbiner et al, 1988]. Additionally, data suggests indirect roles of E-cadherin in mediating assembly and/or disassembly of TJs and desmosomes [van Hengel et al, 1997; Lewis et al, 1997; Tunggal et al, 2005].

E-Cadherin In Epithelial Ovarian Carcinoma.

Downregulation of the cadherin-catenin complex by disruption of junctional integrity or decreased expression plays an important role in typical carcinomas. Disruption of cell-cell contacts, a hallmark of cancer progression, enhances tumor cell proliferation, migration, invasion, and gain of mesenchymal phenotype (via epithelial-to-mesenchymal transition), all of which potentiate tumor metastasis [Hanahan and Weinberg, 2000]. Most carcinomas exhibit a loss of epithelial markers like E-cadherin during early progression and undergo epithelial-to-mesenchymal transition (EMT). Conversely, normal ovary surface

epithelial cell-cell integrity is maintained by Neural (N)-cadherin and epithelial ovarian carcinomas (EOCs) exhibit an early gain of E-cadherin expression and undergo reverse EMT, or mesenchymal-to-epithelial transition (MET) [Maines-Bandiera et al, 1997; Wong et al, 1999, Wong et al, 2002; Imai et al, 2004; Hudson et al, 2008]. Interestingly, this gain-E-cadherin expression/MET is transient, and further progression towards developing stable metastases appears to be dependent on reversal EMT events at the metastatic site, allowing for peritoneal mesothelium adhesion, migration and invasion [Hudson et al, 2008]. Loss of E-cadherin may be achieved through downregulation of gene expression, protein trafficking dysregulation or enzyme-mediated cleavage. A soluble, 80 kDa E-cadherin ectodomain (sE-cad) is found in ascites fluid of patients [Darai et al 1998, Sundfeldt et al, 2001], and remains in contact with the primary lesion and the disseminates, potentially playing a role in gain of the mesenchymal phenotype by disrupting EOC cell aggregates [Symowicz et al., 2007]. E-cadherin has a complex mechanism of trafficking, and can be regulated by exocytosis (transport from the Golgi apparatus to the cell membrane), endocytosis (removal from the cell surface either to be recycled or degraded), stabilization at the membrane and proteolytic cleavage at the membrane; these mechanisms are regulated by at least 24 molecules [Bryant et al., 2004]. The potential non-junctional mechanisms by which E-cadherin enhances metastasis are unknown, and more studies are needed to identify a possible role in signaling and the regulation of E-cadherin trafficking after cleavage.

IV. THE MECHANOBIOLOGY OF THE OVARIAN TUMOR MICROENVIRONMENT

Development and Composition of Ascites Fluid.

A large component of the epithelial ovarian carcinoma microenvironment is the peritoneal membrane, a serous lining of the abdominal wall. The membranous structure supports the abdominal organs, and serves as a conduit for the lymphatic vessels and vasculature and the nerves that supply these organs [Tortora et al., 1984]. The anatomy of the peritoneum from the intravascular space outward includes a capillary endothelium, basement membrane, interstitial space, mesothelial basement membrane and the mesothelial lining of the peritoneal space [Tamsma et al., 2001]. The peritoneum is lined by slow growing, mesoderm-derived epithelial cells that secrete surfactant, proteoglycans and glycosaminoglycans to provide a slippery, non-adhesive surface [Mutsaers et al, 2007]. Disease-free peritoneum is believed to be the primary source of malignant ascites in *in vivo* studies of intraperitoneal-injected ovarian carcinoma and mammary adenocarcinoma metastases [Hirabayashi et al., 1970].

More than two thirds of all cases of EOC simultaneously present with ascites [Gotlieb et al., 1998], and ascites development in progressive (ascites may begin

to develop as early as Stage I of EOC) [Stanojevic et al., 2004; Dembo et al., 1990]) or recurrent (treated or untreated) disease is a poor prognostic sign and is correlated with lack of response to treatment [Gotlieb et al., 1998]. Ascites volume averages 4.9 liters in EOC patients, and ranges from 0.8-15 liters [Holm et al, 1989; Yazdi et al, 1996; McNamara et al, 2000]. This plasma exudate is comprised of cellular components (ovarian cancer cells, lymphocytes), and acellular components including a variety of signaling factors including, but not limited to, lysophosphatidic acid (LPA), vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) [Puiffe et al., 2007; Cowden Dahl et al., 2007]. Although the biologic components of the ascites are increasingly being shown to have major implications in the progression of ovarian cancer via many biochemical signaling pathways [Cowden Dahl et al., 2008; Said et al., 2007], the consequences of activating biomechanical signals have not been investigated in depth. Notably, presence of non-malignant ascites (absence of free-floating tumor cells) is correlated with poor prognosis [Dembo et al, 1990; Chung and Kozuch, 2008], suggesting biomechanical influence of accumulating ascites on ovarian carcinoma cells.

Ascites and Force Development.

Increasing intraperitoneal pressure (correlated with increase in ascites volume) leads to compressive force being exerted on disseminating tumor cells [Henrickson et al, 1980; Gotlieb et al, 1998]. Intraperitoneal fluid pressure of

22.1 mmHg in human patients with tense ascites has been measured [Gotlieb et al, 1998], compared with subatmospheric intraperitoneal fluid pressure in healthy mammals [Henrickson et al, 1980]. In addition to the compressive forces exerted on disseminating ovarian tumor cells as ascites accumulates, these free-floating cells are also subjected to non-laminar shear stress. Physiologically, epithelial cell exposure to shear stress is rare. Only select cell types encounter shear stress-induced forces (e. g. blood and immune cells, vascular and colon endothelium). The mechanical forces generated by shear stress are sensed by integrins and potentiate cell signaling that can modulate genes expression [Alenghat and Ingber, 2002]. In colon carcinoma cells, shear-induced mechanical forces potentiates leukocyte-like cell-rolling and adhesion behavior [Byers et al, 1995; Tozeren et al, 1994] and also mediates cell cycle progression via activation of β-catenin-mediated gene transcription [Avvisato et al, 2007].

Development of ascites also leads to massive abdominal distension, and stretching of the peritoneum [Gotlieb et al., 1998]. This stretching can be described as an applied force (strain) exerted on the peritoneal layers (including the mesothelium), and can be recapitulated in biological studies. Study of the mesothelium (layer of peritoneum proximal to disseminating tumor cells in ascites) under mechanical strain may reveal novel mechanism(s) of tumor cell metastasis. As ascites develops and stretches the peritoneum, vessel walls of the microvasculature are stretched, and prolonged stretching of the vessels are hypothesized to increased vessel size, hyperpermeation [Adair et al., 1990] and

extravasation of fibrinogen which forms a thin stromal lining covering the peritoneal mesothelium [Nagy et al., 1995]. This finding is particularly significant because fibrinogen has been found to be upregulated in the peritoneum and stroma of patients with epithelial ovarian carcinoma, and is suggested to facilitate EOC metastasis [Wang et al., 2005; Olt et al., 1992; Ma et al., 2007]. As ovarian carcinoma progresses, and ascites develops, increasing abdominal pressure leads to abdominal wall (mesothelium) distension. This mechanical stretching may lead to pro-metastatic alterations of the mesothelium, including production of stroma that is conducive to ovarian carcinoma cell adhesion, migration and invasion.

The Biomechanical Force Sensors.

Mechanical sensing has long been studied in cardiac and vascular disease; however, the ability of all cell types to sense and generate mechanical forces is fundamental for cells to respond to their microenvironment [Burridge et al, 1988; Galbraith and Sheetz, 1997]. A delicate balance maintains tensional homeostasis, wherein the extracellular matrix exerts force on cells that respond by reciprocal generation of tensional force [Ingber, 2003a; Ingber, 2003b]. Investigation of mechanobiology in the last twenty years has revealed coupling and crosstalk between cell mechanosensing and biochemical signaling pathways [Wang and Thampatty, 2006]. Disruption of tensional homeostasis by increasing environmental force, or propensity of reciprocal cellular forces may lead to

dysregulation of mechanosensitive biochemical signaling [Makale, 2007]. Epithelial cells sense mechanical stimuli from the extracellular matrix (ECM) and laterally from neighboring cells through integrin-based focal adhesions and cadherin-based adherens junctions, respectively [Bissell and Nelson, 1999; Gumbiner, 1996]. The primary signaling mechanisms of mechanotransduction in epithelial cells are activation of mechanosensitive channels (MSCs) and force-induced integrin signaling [French, 1992; Baumgarten, 2000]. Some MSCs may be permeable to monovalent (sodium, potassium) and/or divalent ions (calcium), and may initiate mechano-regulated cellular responses, such as cytoskeletal rearrangements that potentiate cell migration and invasion [Yang and Sachs, 1990; Baumgarten, 2000]. Integrins mediate bi-directional mechanical signaling across cell membranes. As “outside-in” sensors, ECM-induced integrin leads to FAK-mediated signaling and downstream modifications of cell-matrix and cell-cell adhesion, gene expression, and metabolism [Ingber, 1991]. Interestingly, mechanical stress is in turn induce required for maturation of focal adhesions, composed of clustered integrins and accumulating scaffolding and cytoskeletal proteins [Balaban et al, 2001; Ridley and Hall, 1992].

Recently, studies have demonstrated the propensity for tumor cells to sense and respond to mechanical stimuli, although the responses themselves have not been fully elucidated [Ingber, 2005; Ingber, 2008; Paszek et al., 2006]. Tumor cells are believed to sense mechanical forces (i.e. compression, tensional and shear forces) through the plethora of surface-expressed molecules that regulate

cell-matrix and cell-cell interactions [Bershadsky et al, 2003; Chen et al, 2004]. Endothelial cells can sense fluid shear through a complex that includes vascular endothelial (VE)-cadherin and platelet/endothelial cell-adhesion molecule 1 (PECAM1) [Tzima et al, 2005]. Integrin activation and subsequent “inside-out” biochemical signal modulated-cytoskeletal modifications [Ingber, 2008; Paszek et al., 2006]. Physical coupling of integrins and cadherins to the nucleus via cytoskeleton may provide a mechanism through which changes in the mechanical environment can mediate cell behavior through modifications of gene expression [Wang et al, 2009]. In fact, direct mechanical stimulation of integrins activates protein-translation-complex formation [Chicurel et al, 1998], G-protein signaling [Meyer et al, 2000], and protein kinase activity [Chien et al, 2007; Giannone et al, 2006].

V. WNT SIGNALING ITS POTENTIAL ROLES IN OVARIAN CANCER

The Wnt Signaling Pathway.

The Wnt signaling pathway regulates a diversity of processes fundamental to embryogenesis including proliferation, differentiation, polarity, adhesion and motility [Wend et al, 2010; van Amerongen et al, 2009; Barker et al, 2000]. The highly conserved and complex Wnt pathway transduces signals from the extracellular environment through transmembrane receptors and co-receptors to impact cytoskeletal rearrangements and gene expression changes and thereby modulate cell behavior. In the adult organism, aberrant activation of these same biological processes can induce neoplasia and promote tumor progression [Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2010]. There are two distinct pathways for transduction of Wnt signals: the canonical Wnt/β-catenin pathway and the non-canonical β-catenin independent pathway. The latter can be further subdivided into Wnt/planar cell polarity (PCP) and Wnt/Ca^{2+} signaling pathways.

Canonical Wnt signaling is commonly activated by secreted proteins in the Wnt family, currently consisting of 19 members [Wend et al, 2010; vanAmerongen et

al, 2009; Barker et al, 2000; Wu et al, 2007]. Wnts bind to transmembrane G-protein coupled Frizzled (Fzd) receptors. Interaction of ligated Fzd with co-receptors designated LRP-5 or LRP-6, members of the low-density lipoprotein receptor-related protein (LRP) family, initiates Wnt signaling. In the absence of Wnt signaling, β-catenin functions as a structural component of E-cadherin junctions and is complexed with the cytoplasmic tail of E-cadherin. In normal epithelial cells, the majority of β-catenin is associated with E-cadherin at cell-cell junctions, and the levels are maintained at low concentrations in the cytoplasm by phosphorylation-dependent degradation of β-catenin. Cytoplasmic β-catenin is targeted to a complex comprised of axin, adenomatous polyposis coli (APC) and glycogen synthase kinase 3-beta (GSK3-β), resulting in phosphorylation of β-catenin that targets it for degradation through the ubiquitin/proteasome pathway. Activation of Wnt signaling leads to phosphorylation of LRP-5/6, resulting in recruitment of Axin and Dishevelled (Dvl) to the plasma membrane, thereby functionally disrupting the β-catenin degradation complex. This in turn enables accumulation of cytoplasmic β-catenin which can then translocate to the nucleus, bind proteins in the T-cell factor (Tcf)/lymphoid enhancer factor (Lef) family, and activate transcription of Wnt target genes. Wnt signaling can be inhibited by sequestration of Wnt ligands via interaction with secreted Frizzled-related protein (SFRP) or Wnt-inhibitory factor (WIF-1) as well as by blocking co-receptor binding between Kremen and LRP-5/6 via Dickkopf (Dkk).

Accumulation of non-junctional β-catenin, and in particular nuclear translocation, is used as a surrogate marker for activation of the Wnt/β-catenin pathway. This is seen in a number of human cancers, as summarized below, and often results from mutations in Wnt pathway components. With the exception of the endometrioid histotype, Wnt pathway mutations are rare in ovarian cancer [Wu et al, 2007; Zhai et al, 2002]. However, accumulating evidence suggests a role for Wnt signaling in ovarian tumorigenesis in the absence of genetic mutations, suggesting alternative mechanisms for Wnt pathway activation in EOC [Gatcliffe et al, 2008; Rask et al, 2003; Lee et al, 2003].

Wnt Signaling In Ovarian Cancer.

Progression of most cancers has been associated with activation of Wnt signaling acquired through two major routes: mutations in key components of the pathway, or through mutation-independent aberrant gene expression. Interestingly, many cancers including breast, prostate, lung, thyroid, and pancreas [Howe et al, 2004; Ishigaki et al, 2002; Mazieres et al, 2005; Miyake et al, 2001; Verras et al, 2006; Zeng et al, 2006] do not depend on mutations in APC, Axin, or β-catenin for activation of Wnt signaling. In contrast, the majority of colon, uterine, and bladder tumors are strongly associated with APC, Axin, or β-catenin mutations [Bienz et al, 2005; Kastritis et al, 2009; Oving et al, 2002; Schlosshauer et al, 2000; Shinohara et al, 2001].

Wnt Signaling Activation Independent of Mutations.

The presence of nuclear or cytoplasmic β-catenin in human breast cancer specimens is considered to be a strong indicator of activated Wnt signaling in this malignancy [Gatcliffe et al, 2008]. Multiple pathways, such as phosphorylation of β-catenin by epidermal growth factor receptor (EGFR)/HER2 [Kanai et al, 1995], regulation of GSK3β activity by insulin, insulin-like growth factor-1 (IGF-1) [Playford et al, 2000], integrin-linked kinase (ILK) [Novak et al, 1998], or phosphatidylinositol 3-kinase (PI3K)/AKT [Sharma et al, 2002], and loss of phosphatase and tensin homolog (PTEN) [Persad et al, 2001] or p53 [Liu et al, 2001; Matsuzawa et al, 2001] have been associated with Wnt activation in breast cancers. Interestingly, activation of Wnt signaling in prostate cancer also depends on PTEN, PI3K/AKT [Sharma et al, 2002], and IGF1 [Verras et al, 2005], and can additionally be regulated by the androgen receptor [Truica et al, 2000]. In lung carcinoma, activation of Wnt signaling likely occurs through routes involving overexpression of Dvl [Uematsu et al, 2003] and repression of Wnt antagonists, such as Wnt inhibitory factor-1 (WIF-1) and Dkk [Mazieres et al, 2004]. Activation of Wnt signaling in pancreatic adenocarcinoma has been related to overexpression of Wnt-1 and frizzled-2, which promotes stabilization of β-catenin [Zeng et al, 2006].

Histotype-Dependent Wnt Signaling Activation in Ovarian Carcinoma.

Mechanisms for activation of Wnt signaling in ovarian carcinoma exhibit histotype dependence. Thus, only endometrioid EOC is strongly associated with activating mutations in β-catenin leading to constitutively active Wnt signaling. The majority of low-grade endometrioid ovarian carcinomas often display nuclear immunoreactivity for β-catenin (70% of cases), and these cases often harbor mutations in the β-catenin gene at codons that encode for residues phosphorylated by GSK3β (54 % of cases) [Gamallo et al, 1999]. Several studies confirmed the predominance of nuclear β-catenin and frequent β-catenin gene mutations in endometrioid EOC as well as in cell lines derived from this histotype [Saegusa et al, 2001; Schlosshauer et al, 2002; Wu et al, 2001]. Nuclear β-catenin in low-grade endometrioid EOC also associates with squamous differentiation and correlates with good prognosis and lack of relapse [Gamallo et al, 1999; Hendrix et al, 2006; Schwartz et al, 2003; Zhai et al, 2002]. Moreover, expression of Wnt target genes including FGF9 has been described, suggesting that this pathway is active in endometrioid carcinomas [Hendrix et al, 2006; Schwartz et al, 2003; Zhai et al, 2002]. Mutations in AXIN in cell lines of endometrioid EOC have also been reported [44], however a distinct study found no mutations in either APC or AXIN in human endometrioid EOC [Sarrio et al, 2006]. Further, high-grade endometrioid ovarian carcinomas do not display nuclear β-catenin immunoreactivity and progression is not associated with β-catenin mutations [Gamallo et al, 1999]. This evidence supports the existence of

two distinct subtypes of endometrioid EOC that may originate from different sources, [Bell et al, 2005] based on differences in molecular pathobiology.

In contrast to endometrioid EOC, ovarian carcinomas of serous, clear cell, and mucinous histotypes have only rarely been associated with activating mutations in the key proteins of the Wnt signaling pathway. One report indentified cases of clear cell EOC positive for nuclear β-catenin [Wang et al, 2006]. Another study identified mucinous EOC positive for mutations in the β-catenin gene in the GSK3β binding region [Sagae et al, 1999]. Nevertheless, several lines of evidence implicate activation of Wnt/β-catenin signaling in serous EOC in the absence of activating mutations in either APC, AXIN, or β-catenin. The strongest evidence is the presence of nuclear β-catenin. A broad range of (3-59%) of serous EOCs have been reported to contain nuclear and cytoplasmic β-catenin [Wang et al, 2006; Karbova et al, 2002; Lee et al, 2003]. It is noteworthy that a significantly higher percentage of high-grade (23%) serous EOC correlated with the presence of nuclear β-catenin compared to low grade (2.1%) [Lee et al, 2003], opposite from trends observed for endometrioid EOC [Gamallo et al, 1999]. Together these observations suggest that factors other than mutations initiate Wnt signaling activation in serous EOC progression.

Microenvironmental Activation of the Wnt Pathway.

Intraperitoneal dissemination provides a unique microenvironment for ovarian carcinoma metastases. Progression of EOC is hallmarked by shedding of single and multi-cellular aggregates (MCAs) of malignant epithelial cells from the primary tumor [Hudson et al, 2008; Barbolina et al, 2009]. Accumulation of malignant ascites in the peritoneal cavity is common, particularly in women with late stage EOC. Thus, metastasizing EOC cells exist in a milieu rich in inflammatory cells [Freedman et al, 2004] and growth/signaling factors, including vascular endothelial growth factor (VEGF) [Zebrowski et al, 2999; Kraft et al, 1999; Santin et al, 1999], epidermal growth factor (EGF) [Miyamoto et al, 2004], transforming growth factor (TGF) [Saltzman et al, 1999; Abendstein et al, 2000] family members and lysophosphatidic acid (LPA) [Puiffe et al, 2007], providing ample opportunity for cross-talk between signaling networks. Ascites accumulation also modifies peritoneal mechanobiology, altering the force environment of both metastatic tumor cells and peritoneal mesothelium [Esquis et al, 2006; Henriksen et al, 1980]. Formation of secondary tumors at peritoneal organs (colon, omentum, uterus, liver) is achieved by β1-integrin-mediated anchoring to the mesothelium and submesothelial matrix [Ellerbroek et al, 1999; Ellerbroek et al, 2001; Barbolina et al, 2007; Lessan et al, 1999], representing a significant transition from a free-floating cell or aggregate to a three-dimensional matrix-anchored structure. Within this unique metastatic niche, current evidence suggests multiple opportunities for regulation of Wnt signaling via molecular, mechanical, and adhesion-dependent microenvironmental cues.

VI. PROJECT RATIONALE AND CENTRAL HYPOTHESIS

Rationale.

Ovarian carcinoma is the number one cause of death by gynecologic cancers in the United States. With a 5-year survival of less than 30% in women diagnosed with metastatic disease, the need for elucidation of the mechanism of metastasis and development of drugs to prevent metastatic disease is clear. Ovarian carcinoma arises from the epithelial layer of the ovary, dedifferentiating as the carcinoma progresses and metastasizes by shedding from the ovary into the peritoneal cavity. Within this cavity, epithelial ovarian carcinoma (EOC) cells exist as single cells and in small clusters known as multicellular aggregates (MCAs) and experience shear, strain (stretch) and compressive forces exerted upon them by ascites, a malignant fluid that develops as the cancer progresses. In a novel pattern of transition, epithelial ovarian carcinoma cells undergo an early transition to an epithelial phenotype characterized by gain expression of E-cadherin, the primary component of adherens junctions (AJs). Late in the metastatic process EOC cells experience a reverse transition (losing E-cadherin expression) and regain the mesenchymal phenotype, which facilitates their anchoring to and invasion through the peritoneal mesothelium and

submesothelial matrix. As adherens junctions are dissolved and E-cadherin surface expression is lost, β-catenin is freed from the cell surface. In addition to stabilizing AJs, β-catenin also modulates gene expression and is tightly regulated by a GSK3-β/Axin/APC degradation complex. The fate of freed β-catenin following AJ dissolution in ovarian carcinoma is unknown. The experiments in this project are designed to investigate the **central hypothesis** (**Fig. 1.3**) that microenvironmental factors (integrin engagement, lysophosphatidic acid (LPA), and mechanical force) lead to aberrant, mutation-independent activation of Wnt signaling and pro-metastatic modifications of ovarian carcinoma cells, which potentiate metastatic success.

Models.

Four ovarian carcinoma cell lines are used in these studies: OVCA429, OVCA433, DOV13, and SKOV3ip (**Fig. 1.4**, Experimental Methods). OVCA429 and OVCA433 were chosen for their high expression of E-cadherin, which correlates to the gain of E-cadherin expression during early tumor progression. Due to the novelty of these mechanical force studies, this work attempts to evaluate a broad range of cellular responses, including changes in protein activity and expression, cell proliferation and gene expression. As disseminating ovarian tumors are heterogenous in nature, the experiments in this chapter utilize both E-cadherin-expressing cell lines (OVCA429 and OVCA433) and N-cadherin expressing cell lines (DOV13 and SKOV3ip).

Specific Aims.

Specific Aim 1 (Chapter 2) investigates the role(s) of ascites-induced increased intraperitoneal forces on disseminating ovarian tumor cells. ***Specific Aim 2 (Chapter 3)*** seeks to elucidate the fate of accumulating cytoplasmic β-catenin following lysophosphatidic acid (LPA) treatment, and whether nuclear accumulation of β-catenin leads to gain of mesothelial phenotype and/or adhesive and invasive potential. ***Specific Aim 3 (Chapter 4)*** investigates β1 integrin engagement-induced accumulation of free cytoplasmic β-catenin after disruption of adherens junctions. ***Specific Aim 4 (Chapter 5)*** investigates the potential for pathway crosstalk between LPA – LPA receptor signaling and integrin clustering and activation.

1.1 STRUCTURE OF LPA

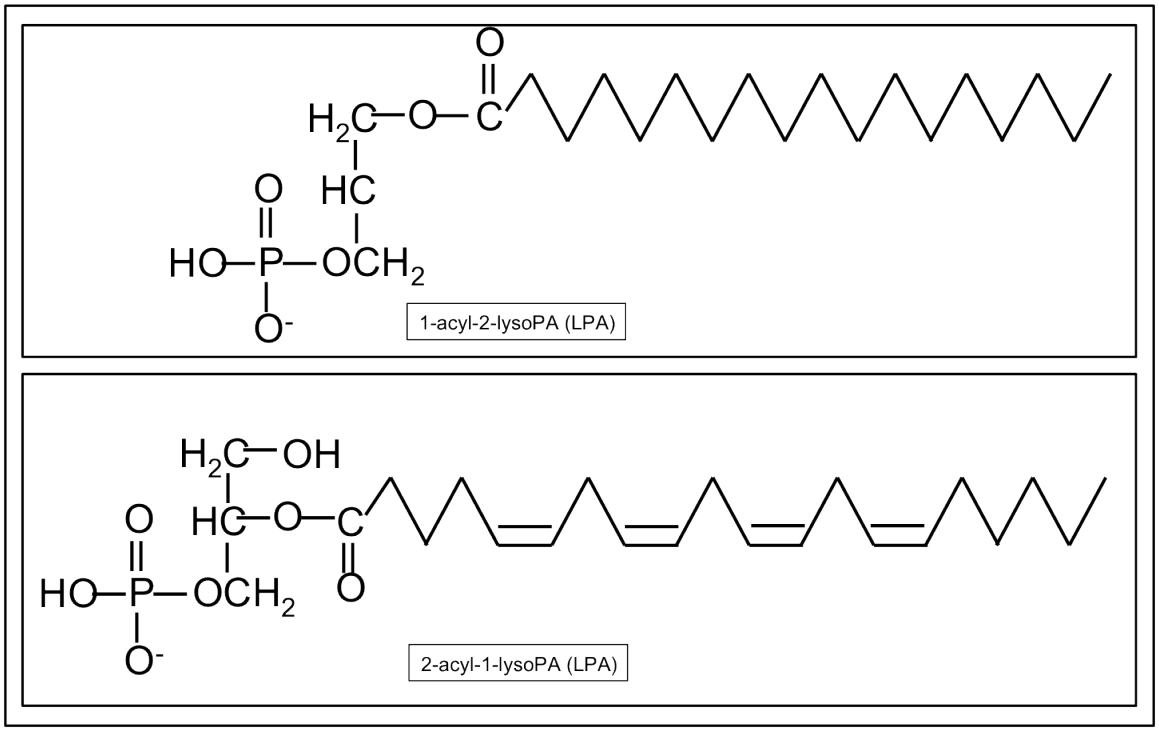


Figure 1.1: Structure of Lysophosphatidic Acid. *sn-2* (*A*) and *sn-1* (*B*) isoforms of LPA are depicted. 1-Oleoyl LPA is an *sn-1* (18:1) LPA.

1.2 INTEGRIN SIGNALING

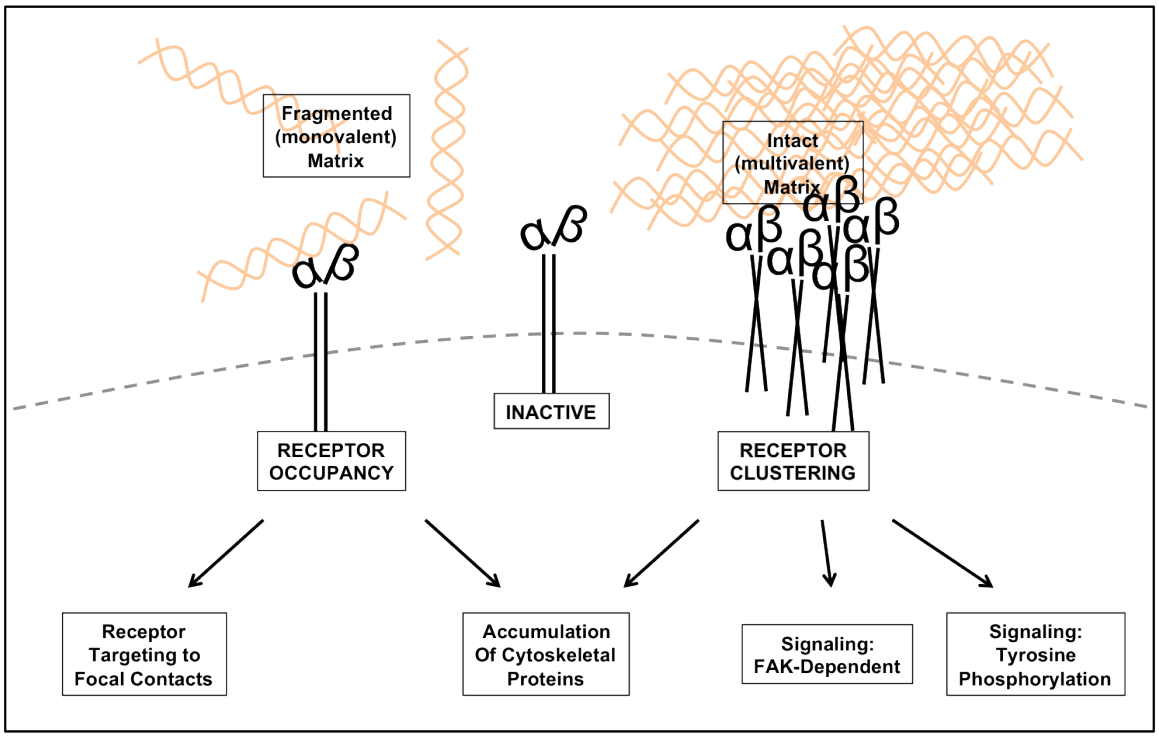


Figure 1.2: Integrin Signaling. Differential signaling responses can be elicited by substrate ligation of fragmented matrix compared with intact matrix-induced integrin clustering.

1.3 CENTRAL HYPOTHESIS

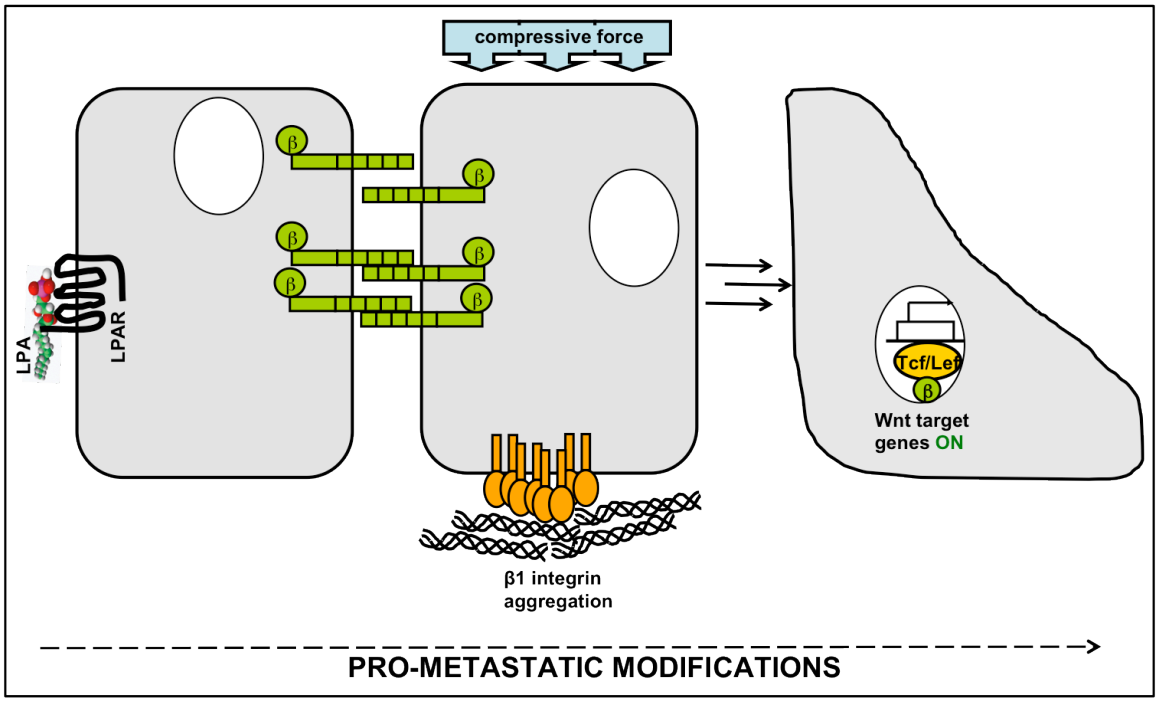


Figure 1.3: Central Hypothesis. Microenvironmental factors (compressive force, LPA and integrin clustering) aberrant, mutation-independent activation of Wnt signaling and pro-metastatic modifications of ovarian carcinoma cells, which potentiate metastatic success.

1.4 CELL LINE MODELS

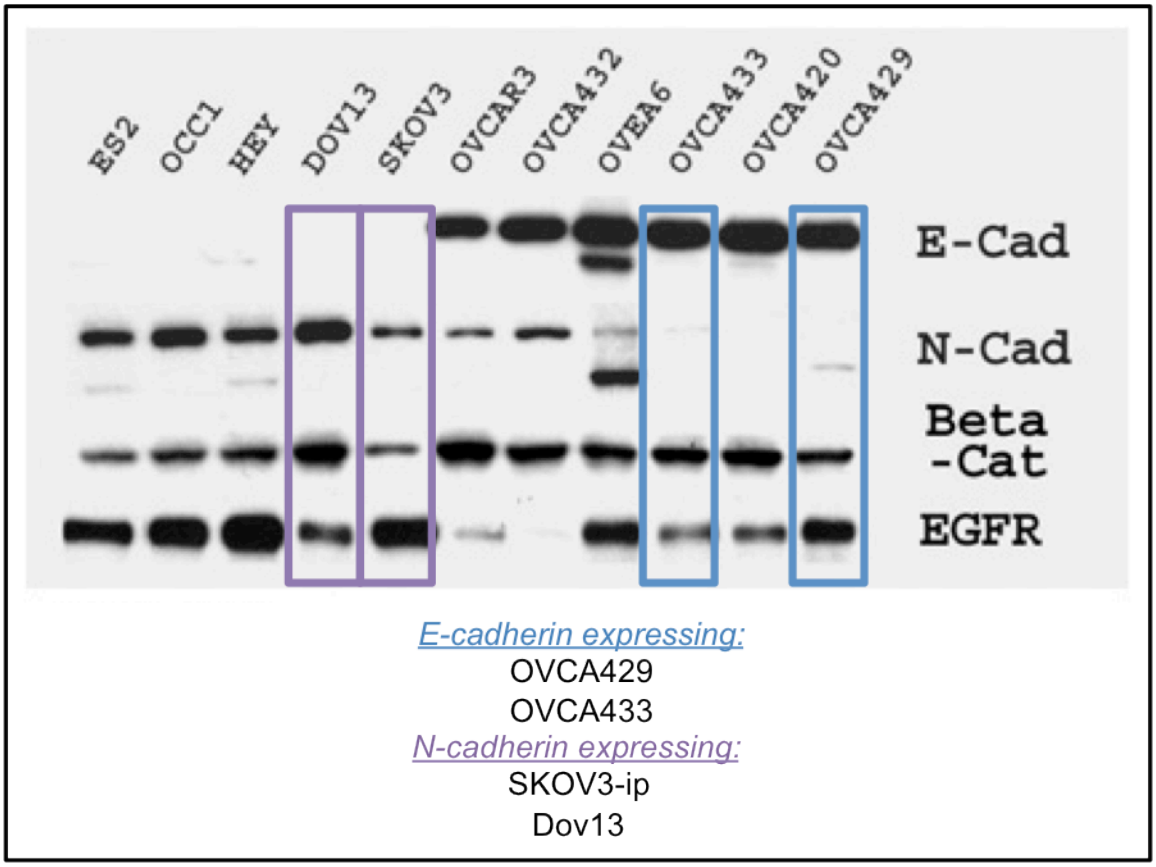


Figure 1.4: Cell Line Models. In order to investigate mechanisms of E-cadherin loss in disseminating ovarian tumor cells, two E-cadherin-expressing cell lines, OVCA429 and OVCA433 were utilized (*blue*). The role of compressive force in ovarian tumors is evaluated in both N-cadherin- (*purple*) and E-cadherin- (*blue*) expressing cell lines.

2. INTRAPERITONEAL PRESSURE MODULATES TUMOR CELL BEHAVIOR

I. RATIONALE

More than two-thirds of all cases of EOC simultaneously present with malignant ascites. Ascites development in progressive or recurrent disease is a poor prognostic indicator and is correlated with lack of response to treatment [Gotlieb et al, 2002; Dembo et al, 1990; Stanojevic et al, 2004]. In comparison to the peritoneal cavity of disease-free women that contains between 5 and 20 ml of peritoneal fluid [Shen-Gunther and Mannel, 2002], ascitic volumes average 4.9 liters in EOC patients, with a range of 0.8-15 liters [Holm et al, 1989; Yazdi et al, 1996; McNamara et al, 2000]. Although biologic components of ascites are increasingly shown to influence progression of ovarian cancer via diverse signaling pathways [Cowden Dahl et al, 2008; Said et al, 2007], potential biomechanical signals activated by increasing intraperitoneal fluid pressure have not been investigated.

Ovarian cancer patients often present with distension of the abdominal cavity, necessary to accommodate ascites fluid [Dembo et al, 1990; Ayantunde and Parsons, 2007]. Functionally, distension is achieved through physical stretching of the compliant peritoneum [Breton et al, 2008]. However, as the peritoneum reaches its elastic threshold, fluid pressure within the cavity increases from subatmospheric to levels as high as 22.1 mmHg [Henrickson et al, 1980; Gotlieb et al, 1998]. Disseminating EOC cells are subsequently subjected to this increase in fluid pressure, in addition to non-laminar shear stress exerted on the free-floating cells.

Tumor cells sense alterations in the force environment via mechanosensing cell surface-expressed proteins, including integrins, and subsequent “inside-out” integrin signaling induces cytoskeletal modifications the effect cell behavior [Ingber et al, 2008; Paszek et al, 2005; Thamilselvan et al, 2004; Ingber et al, 2005]. Force modulation in the context of three-dimensional matrix rigidity is under investigation in several other tumor types [Gotlieb et al, 2005; Paszek et al, 2005; Butcher et al, 2009; Condeelis et al, 2003; Wyckoff et al, 2006], however the potential effects of the complex mix of strain, compression and shear forces conferred by ascites fluid are unknown.

In this chapter, the peritoneal cavity is modeled as a simple fluid-filled sac exposed to increased fluid pressure. Based on the premise that increasing

abdominal pressure leads to compression of ovarian tumor cells, the following experiments test the ***hypothesis*** that increased fluid pressure leads to pro-metastatic genotypic and phenotypic alterations. Namely, mechano-induced modifications in cell-cell and cell-matrix adhesion molecule expression, pro-metastatic gene expression, and altered protease expression and activity are investigated.

II. RESULTS

Modeling the Peritoneal Cavity.

Previous models of peritoneal dissemination have used extracellular matrix components and mesothelial cells cultured in tissue culture dishes, often adding tumor cells as single cells and multicellular aggregates formed by hanging drop method [Kelm et al, 2002], and measuring downstream events. Very little is known, however, about the cellular dynamics of free-floating cells. In these studies the peritoneal cavity is modeled in its simplest form, as a fluid-filled sac (**Fig. 2.1A-D**). Culture of ovarian tumor cell lines in sterile low-density virgin polyethylene bags facilitates formation of multicellular aggregates, MCAs, (**Fig. 2.2**), which are morphologically similar to aggregates formed by the previously utilized hanging drop method (**Fig. 2.3**).

Characterization of High Fluid Pressure-Induced Cellular Modifications.

Cells were harvested from two-dimensional (2D) culture by trypsinization, and seeded at high density in sterile bags (2×10^6 cells/ml; 12 mls suspension per

bag) using a heated bag sealer (**Fig. 2.1A,B,C**). Cells were allowed to form multicellular aggregates overnight by incubation at 37°C in 5% CO_2 (**Fig. 2.1D**), prior to adding sealed bags to two pre-heated (37°C) cylindrical pressure vessels filled with water (**Fig. 2.4A**). One sealed vessel served as a control, while the second vessel was pressurized by connection to the Instron 8215, a servohydraulic testing system (**Fig. 2.4B**), using a hydraulic pump, and pressure was loaded via displacement of: 1) any residual air in the pump or pressure vessel and 2) water within the pump itself. Pressure containment was achieved by closing two valves, one allowing connection to the Instron and a second valve necessary for evacuating any air prior to pressure load. Both the control and pressurized pressure vessels were incubated in a 37°C water bath during the 8 hour experiment. Following the 8-hour incubation, cells were harvested from the bags and trypsinized for 10 minutes in order to disaggregate cells for counting. Since the same number of cells were seeded in each bag, counted cells from control and test populations were compared directly. Differences in population following pressurization are attributed to changes in the rate of proliferation. High intraperitoneal pressure led to increased proliferation in OVCA433 cells, and no change in OVCA429 cell populations (**Fig. 2.5A**). A trend towards an increase in proliferation in SKOV3ip cells was observed; however, in DOV13 cell populations proliferation was hindered by increased fluid pressure (**Fig. 2.5B**).

Regulation of Proteinase Activity by Compressive Force.

A large volume of literature supports protease-dependent tumor metastasis of numerous tumors, including ovarian cancer [reviewed in Koblinski et al, 2000]. Previous studies have demonstrated increased urokinase-type plasminogen activator (uPA) activity in ovarian carcinoma in response to microenvironmental changes [Gil et al, 2008]. uPA activity was measured by colorimetric plasminogen activation assay (as described in Experimental Methods) following high fluid pressure culture, and was unchanged in all four cell lines (**Fig. 2.6**).

Compressive Force Regulates Cadherin Dynamics.

A hallmark characteristic of disseminating epithelial ovarian carcinoma (EOC) is an early gain of E-cadherin followed by a subsequent loss of E-cadherin expression. Interestingly, there are also sub-populations exhibiting heterogenous cadherin expression (N- and E-cadherin expression) [Hudson et al, 2008]. Further, cells at metastatic sites have been shown to expression N-cadherin, E-cadherin, and in some cases both [Hudson et al, 2008; Sarrio et al, 2006; Marques et al, 2004]. Utilizing cell lines expressing N-cadherin (DOV13, SKOV3ip) or E-cadherin (OVCA429, OVCA433), compressive force was applied by increasing fluid pressure, as described previously and in Experimental Methods. E-cadherin expression is increased following pressurized-culture in OVCA429 and OVCA433 (**Fig. 2.7A**). N-cadherin expression is nearly doubled in both DOV13 and SKOV3ip cells cultured under increased fluid pressure (**Fig. 2.7B**).

Mechanical Deformation Regulates Gene Expression in Ovarian Carcinoma.

It has been postulated that one consequence of altering environmental mechanics, and thus cellular cytoskeletal structure, is deformation of the nucleus that may modulate gene expression [Huang and Ingber, 1999]. Interestingly, mechanical compression of colon tissue leads to nuclear translocation of transcriptionally-active β-catenin [Whitehead et al, 2008]. Here, three β-catenin target genes (*MMP9, SNAI1, WNT5A*) have been chosen based on literature suggesting their role(s) in ovarian carcinoma progression. MMP-9 protein expression is high expression in all four ovarian carcinoma histotypes [Symowicz et al, 2007] and is increased 2-fold in malignant ascites [Jacobs and Menon, 2004]. Further, the pro-metastatic roles MMP-9 in extracellular matrix remodeling have been intensely studied in ovarian cancer [Ellerbroek et al, 1999; Davidson et al, 1999; Huang et al, 2002]. Previous data have also suggested a role for proteases in down-regulation of surface-expressed E-cadherin [Symowicz et al, 2007; Liu et al, in press].

In order to evaluate potential mechanical regulation of *MMP9* gene expression, RNA isolation was performed in control and test samples and evaluated by RT-PCR as described in Experimental Methods. Interestingly, *MMP9* mRNA was increased in DOV13 (p-value= 0.0292) and trended toward increase in OVCA429 (p-value= 0.0949) and OVCA433 (p-value= 0.0528) (**Fig. 2.8**). Increased fluid

pressure did not affect *MMP9* expression in SKOV3ip (**Fig. 2.8**). Phenotypic plasticity, as characterized by gain and/or loss of E-cadherin, is a fundamental attribute of ovarian carcinomas [Hudson et al, 2008]; therefore, understanding the mechanisms that regulate plasticity may in turn elucidate mechanisms of metastatic success. As a regulator of E-cadherin gene expression, *SNAI1* mRNA levels were evaluated in the E-cadherin-expressing cell lines, OVCA429 and OVCA433. It both cell lines *SNAI1* mRNA expression was induced over 30-fold (**Fig. 2.9**, OVCA429 – p-value=0.0462; OVCA433 – p-value=0.0166). The Wnt signaling ligand Wnt5a is expressed in the normal human ovary epithelium [Bitler et al, 2011], and its dysregulation has been suggested to correlate with poor overall prognosis [Peng et al, 2011]. Evaluation of *WNT5A* mRNA expression following increased fluid pressure revealed increased mRNA levels in OVCA433 (p-value= 0.0477) and a trend towards upregulation in SKOV3ip, DOV13 (p-value= 0.1835), and OVCA429 (p-value= 0.1618) (**Fig. 2.10**).

Wnt5a is Expressed in Ovarian Carcinoma and Mediates Cell Behavior.

Increased *WNT5A* mRNA in response to increased fluid pressure (**Fig. 2.10**) supports others' findings that Wnt5a, a secreted signaling protein, plays a role in ovarian tumor progression [Peng et al, 2011]. In order to identify expression of Wnt5a protein in ascites fluid, human ascites samples from 1 normal, 6 benign, and 33 EOC (two Stage I, 21 Stage III, and ten Stage IV) patients were evaluated for protein expression. Ascites samples were run on 9% SDS-PAGE gels,

transferred to PVDF membranes and immunoblotted with an anti-Wnt5a antibody (R&D Systems; 1:1000). As predicted, Wnt5a expression tended to be higher in tumor ascites samples compared with benign and normal with band intensity greater in Stage III and Stage IV samples compared with Stage I (**Fig. 2.11**, 49 kDa, black *dashed arrow*). Interestingly, there is strong anti-Wnt5a immunoreactivity in the normal sample (*red arrow*). One explanation for this result may be cycling Wnt5a levels in pre-menopausal menstruating women. A benign sample from a patient with ovarian hyperstimulation syndrome (OHSS) also exhibits high Wnt5a expression (*black solid arrow*), which is a typical observation in OHSS [Jansen et al, 2004].

The Western blot Wnt5a protein expression data was also validated by a more sensitive Wnt5a ELISA experiment (**Fig. 2.12**), and the quantitative expression values correlated directly with immunoblot band intensity (range: 13-3622 ng/ml). Stage IV ascites samples had an average Wnt5a expression of 516.8 ng/ml, higher than those of Stage III (402.7 ng/ml), Stage I (263.5 ng/ml), benign (11.6 ng/ml, value from OHSS patient was omitted from the calculation), and normal (317 ng/ml) (**Table 2.1**). In addition to evaluating ascites samples, whole cell lysates from four ovarian carcinoma cell lines (OVCA429, OVCA433, DOV13, SKOV3ip) were evaluated for Wnt5a expression. The highest Wnt5a expression was observed in OVCA433 (627 ng/ml) followed by DOV13 (428 ng/ml), SKOV3ip (199 ng/ml) and OVCA429 (164 ng/ml) in descending order (**Table 2.2**).

In order to evaluate the functional consequence(s) of high Wnt5a expression on EOC cell metastasis, adhesion of OVCA429, OVCA433, DOV13 and SKOV3ip to Collagen type I (10 μg/ml) was analyzed following 24-hour Wnt5a treatment. Cells were treated with PBS or 400 ng/ml Wnt5a (correlates to median and mean of Wnt5a expression in ascites), and allowed to adhere to Collagen type I matrix for 30 minutes (OVCA429, OVCA433) or 90 minutes (DOV13, SKOV3ip). Wnt5a-treated SKOV3ip cells respond robustly, with 60% greater cells adhering to Collagen type I (p-value=0.0036). OVCA429 (p-value= 0.0695) and OVCA433 (p-value= 0.2307) trend towards increased adhesion following Wnt5a treatment. Adhesion of DOV13 cells was unchanged, p-value= 0.5866 (**Fig. 2.13**).

2.1 MODELING THE PERITONEAL CAVITY AS A FLUID-FILLED SAC

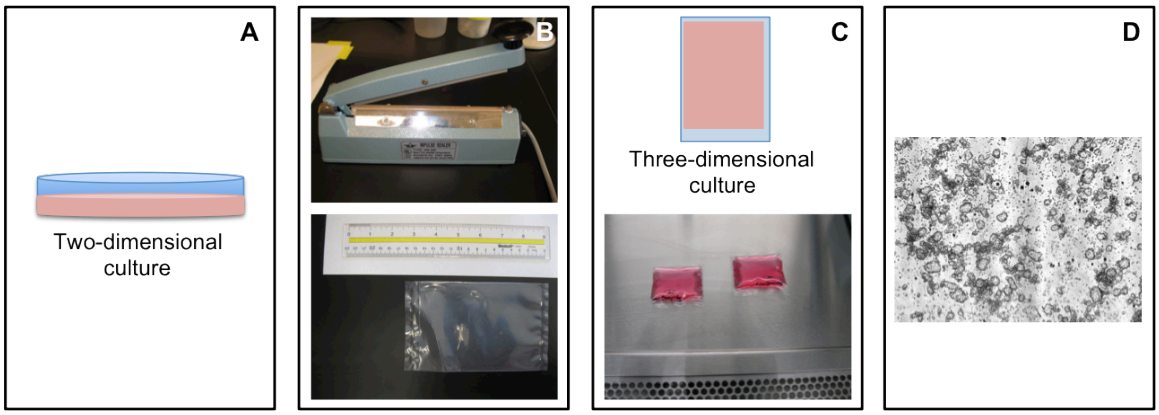


Figure 2.1: Modeling the Peritoneal Cavity as a Fluid-Filled Sac. Cells are harvested from two-dimensional culture (*A*) and seeded at high density into sterile stomacher bags (*B,C*). Bags are sealed using a heated bag sealer (*B*), and allowed to form multi-cellular aggregates overnight (*D*).

2.2 MULTICELLULAR AGGREGATE FORMATION IN FLUID-FILLED SACS

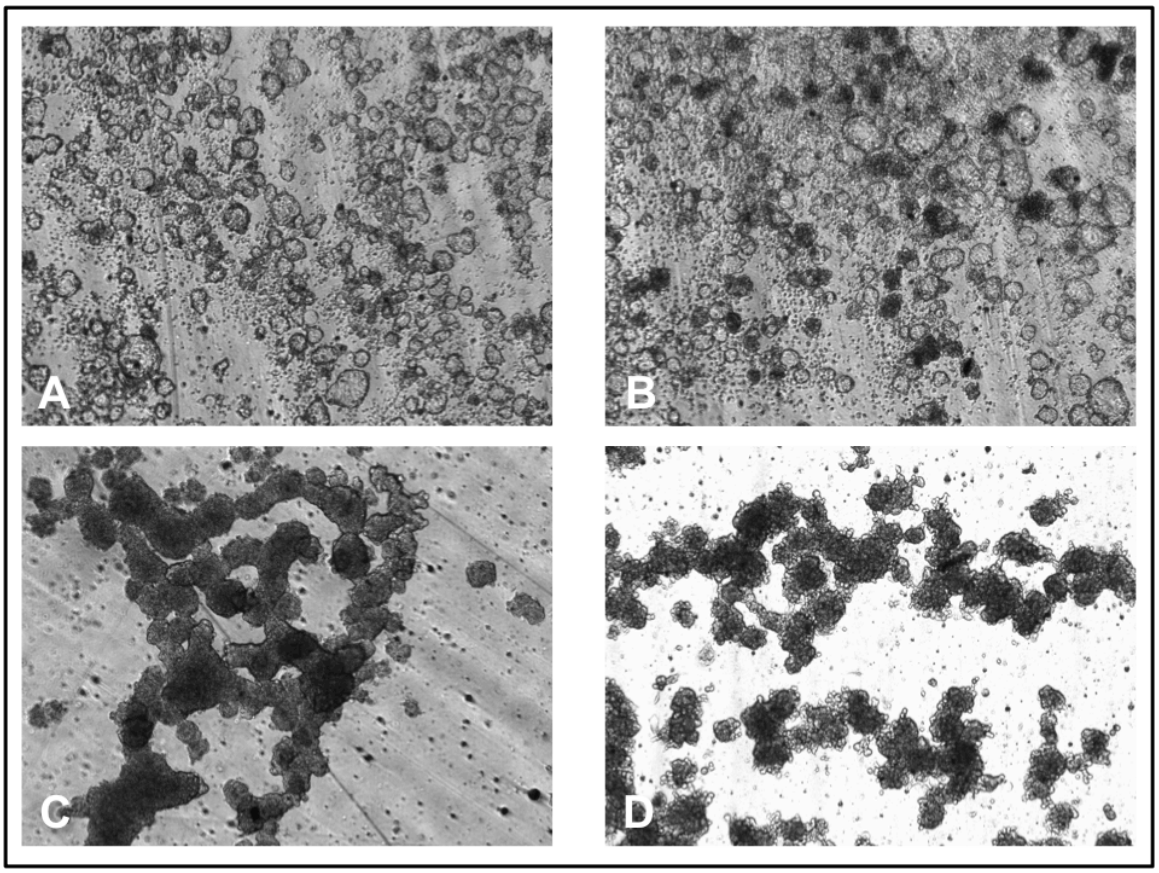


Figure 2.2: Multi-Cellular Aggregate Formation in Fluid-Filled Sacs. Each of the four cell line models, OVCA429 (*A*), OVCA433 (*B*), DOV13 (*C*) and SKOV3ip (*D*) form aggregates when seeded at 2 million cells per milliliter in sterile stomacher bags.

2.3 COMPARISON OF FLUID-FILLED SAC MODEL TO HANGING DROP METHOD

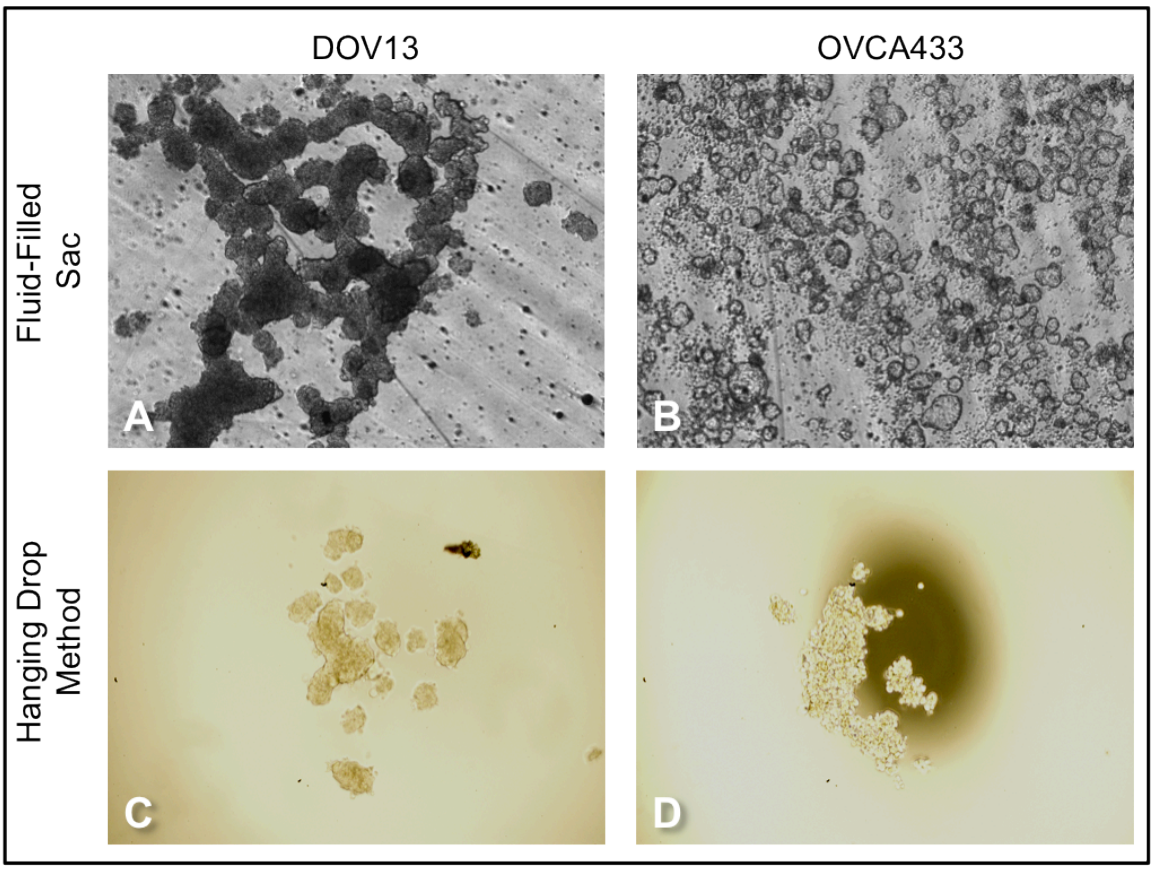


Figure 2.3: Comparison of Fluid-Filled Sac Model to Hang Drop Method. DOV13 (*A,C*) and OVCA433 cells (*B,D*) form morphologically similar multi-cellular aggregates (MCAs) in two models of MCA formation.

2.4 MODELING INTRAPERITONEAL FLUID PRESSURE

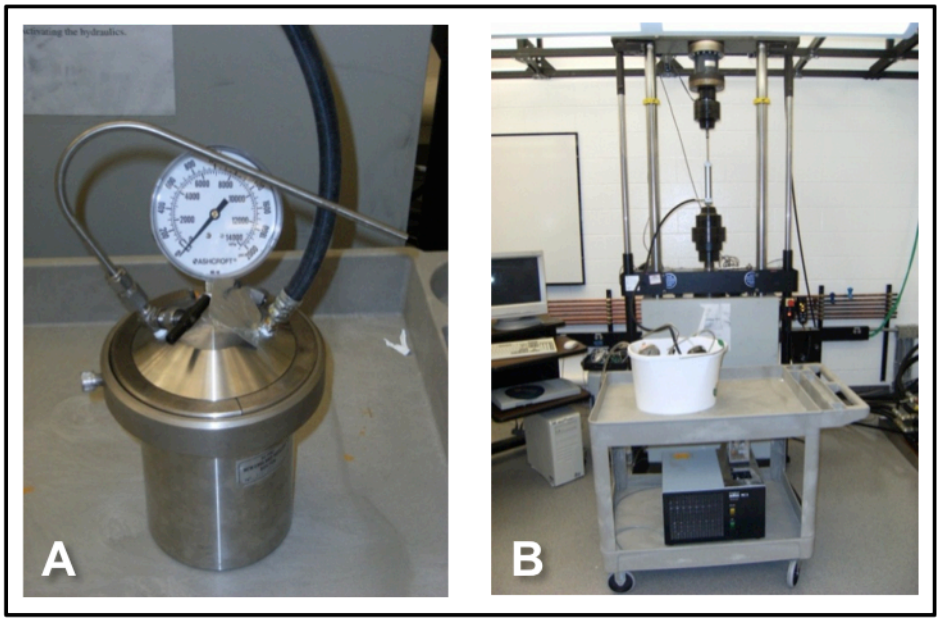
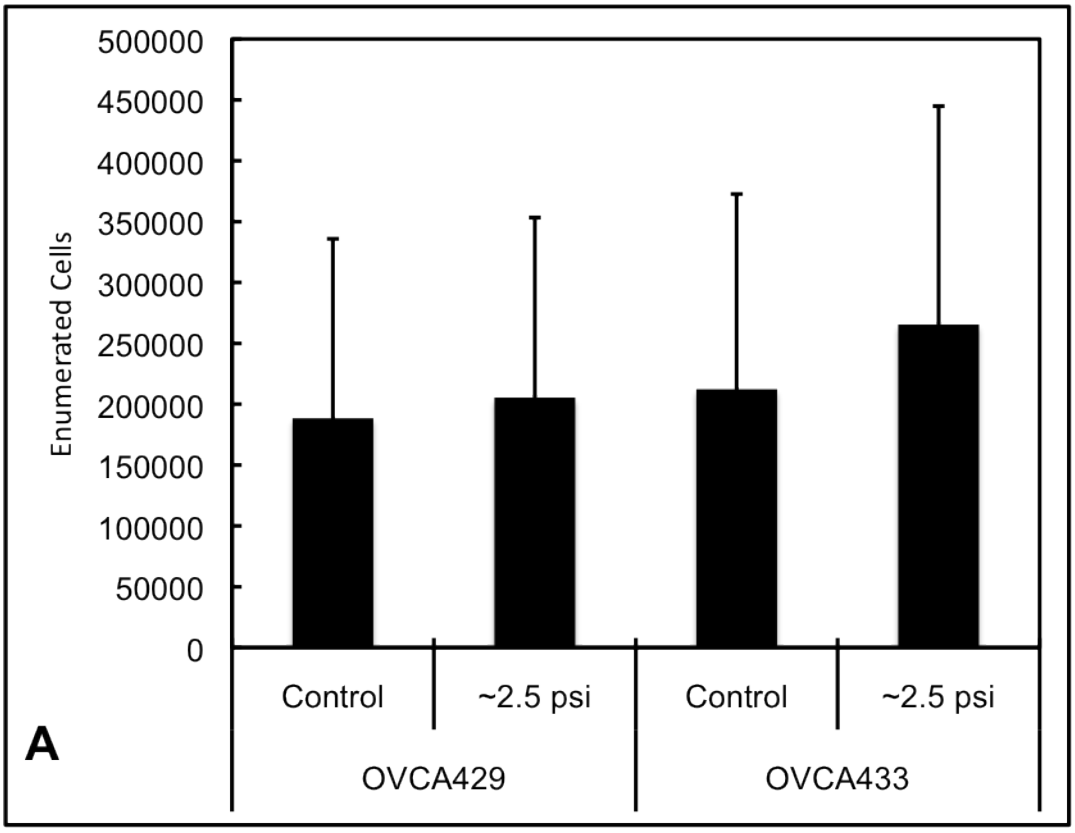


Figure 2.4: Modeling Intraperitoneal Fluid Pressure. Pressurized canister used for applying increased pressure to cells (*A*). Instron 8215 servohydraulic system compresses water to increase pressure in canister above atmospheric levels (*B*).

2.5 HIGH FLUID PRESSURE MODULATES CELL PROLIFERATION



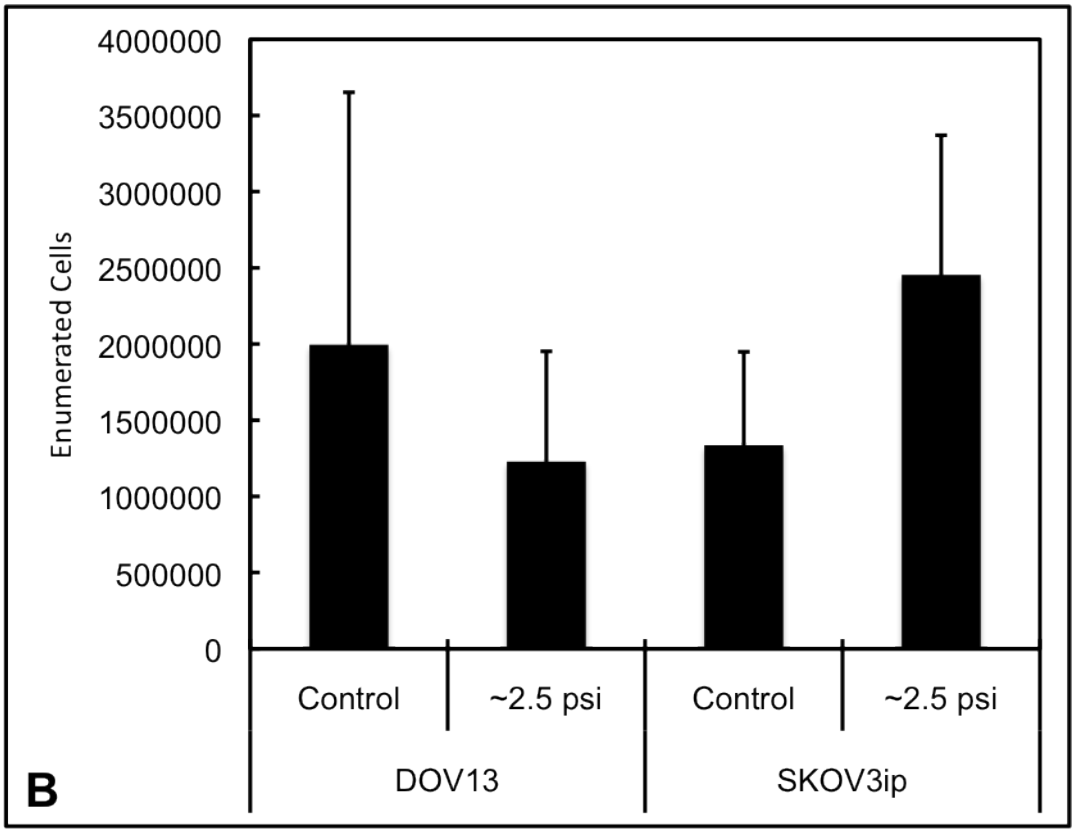


Figure 2.5: High Fluid Pressure Modulates Cell Proliferation. Cells were seeded ($2x10^6$) into bags and allowed to form multicellular aggregates (MCAs) overnight. Bags were transferred to pressure vessels and 25 mmHg fluid pressure was applied for 8 hours. MCAs were subsequently disaggregated by 0.25% trypsin-EDTA, and enumerated using a cell counter in control and pressure-cultured samples for each cell line. A) OVCA433 cell populations tended to increase slightly in response to high fluid pressure; OVCA429 cell populations were unchanged. B) Populations of SKOV3ip also trended towards a large increase following culture under increased fluid pressure, while DOV13 cell populations decreased.

2.6 INCREASED FLUID PRESSURE DOES NOT AFFECT UPA ACTIVITY

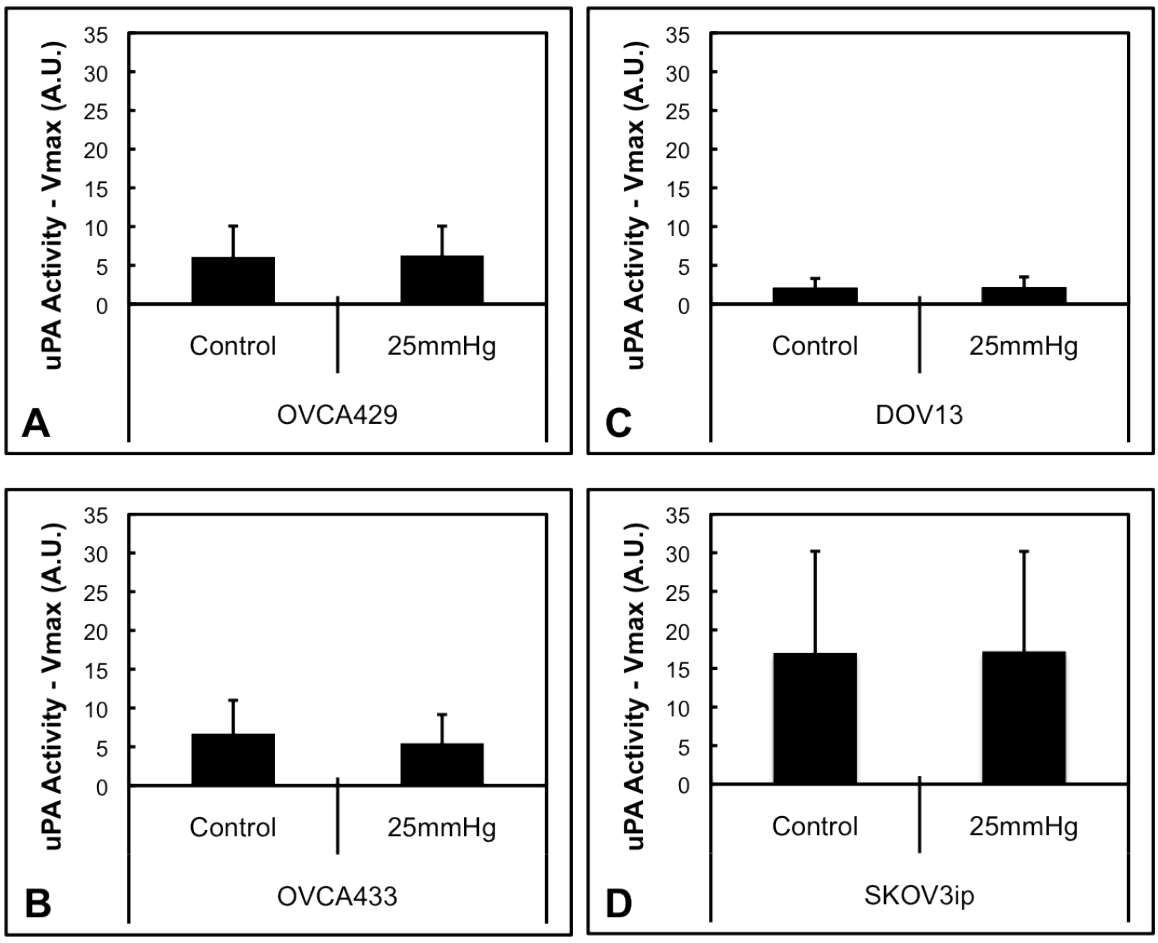


Figure 2.6: Increased Fluid Pressure Does Not Affect uPA Activity. Conditioned medium was collected from control and pressure-cultured cells and uPA activity was evaluated by colorimetric plasminogen activation assay. Conditioned medium was diluted in 20 mM HEPES buffer, and incubated with plasminogen (Pg) and Val-Leu-Lyspara-nitroanilide (VLK-pNA). uPA activates Pg, which in turn cleaves VLK-pNA to VLK, NA and Pm. NA absorbs at 405nm and its formation is measured using a microtiter plate reader. Results reveal no significant change in substrate processing, a measure of activity, in all cell lines (*A-D*).

2.7 CADHERIN EXPRESSION IS UPREGULATED FOLLOWING EXPOSURE TO INCREASED FLUID PRESSURE

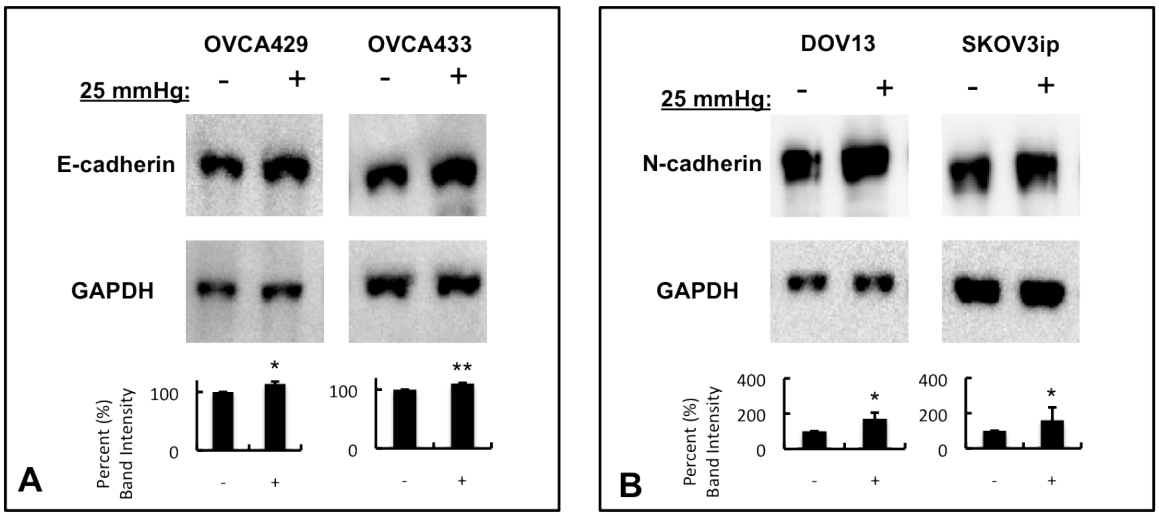


Figure 2.7: Cadherin Expression is Upregulated Following Exposure to Increased Fluid Pressure. Cells were cultured in sterile stomacher bags, in either a pressurized (25 mmHg) or control vessel for 8 hours. Whole cell lysates were collected in mRIPA buffer, then analyzed for N- or E-cadherin expression in DOV13 and SKOV3ip or OVCA429 and OVCA433, respectively, by gel electrophoresis (9%) and Western blot (anti-N-cadherin: 1:1000; anti-E-cadherin: 1:1000). A) E-cadherin expression is increased ~10% in both OVCA429 and OVCA433 in cells cultured under pressure, compared with control. B) Pressure-cultured DOV13 and SKOV3ip cell expression of N-cadherin nearly doubled compared with control cultures. Quantitative data represents band intensity of a mean of 3 experiments.
*$p<0.001$, **$p<0.02$

2.8 INCREASED FLUID PRESSURE-CULTURED CELLS EXHIBIT INCREASED *MMP9* MRNA EXPRESSION

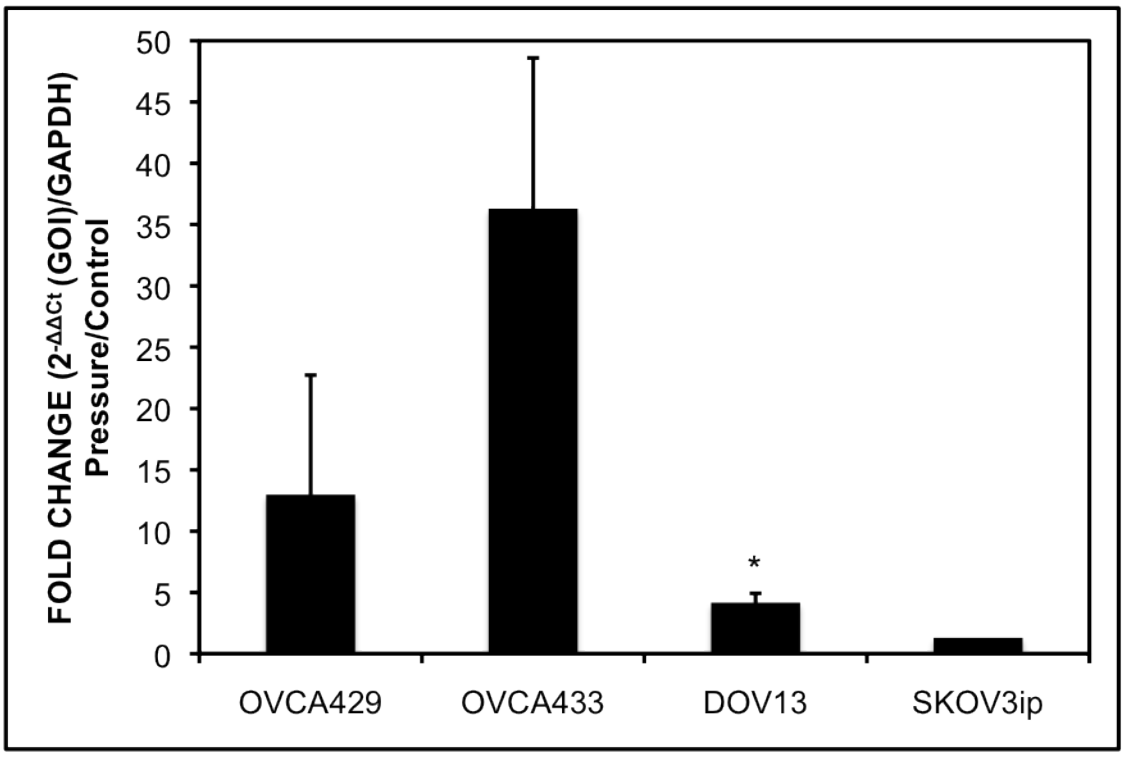


Figure 2.8: Increased Fluid Pressure-Cultured Cells Exhibit Increased *MMP9* mRNA Expression. Cells were cultured in pressure vessels with 25 mmHg fluid pressure for 8 hours, control vessels were not pressurized, prior to isolation of RNA using TriZol and evaluation of Wnt target gene expression by real time RT-PCR as described in Experimental Methods. *MMP9* mRNA expression is increased in OVCA429 (~13-fold), OVCA433 (36-fold), and DOV13 (4-fold) following 8 hour exposure to increased fluid pressure, compared with control cell populations. *MMP9* mRNA expression was unchanged in SKOV3ip cells. Results represent the mean of a minimum of three independent experiments.
*$p<0.03$

2.9 INCREASED FLUID PRESSURE-CULTURED CELLS EXHIBIT INCREASED *SNAI1* MRNA EXPRESSION

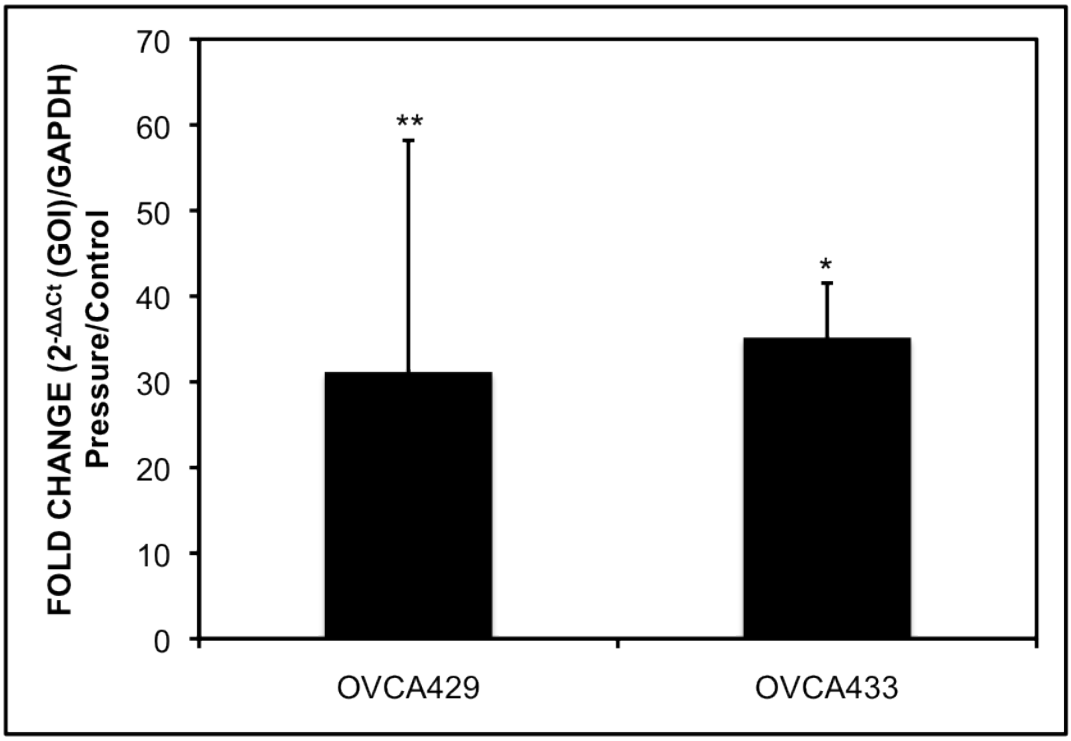


Figure 2.9: Increased Fluid Pressure-Cultured Cells Exhibit Increased *SNAI1* mRNA Expression. Cells were cultured in pressure vessels (control: atmospheric pressure; increased fluid pressure: 25 mmHg) for 8 hours prior to isolation of RNA in Trizol and evaluation of Wnt target gene expression by real time RT-PCR as described in Experimental Methods. *SNAI1* mRNA expression was increased following 8 hour exposure to increased fluid pressure in OVCA429 (31-fold) and OVCA433 (35-fold). Results represent the mean of a minimum of three independent experiments.
*p<0.02, **p<0.05

2.10 INCREASED FLUID PRESSURE-CULTURED CELLS EXPRESS *WNT5A* MRNA

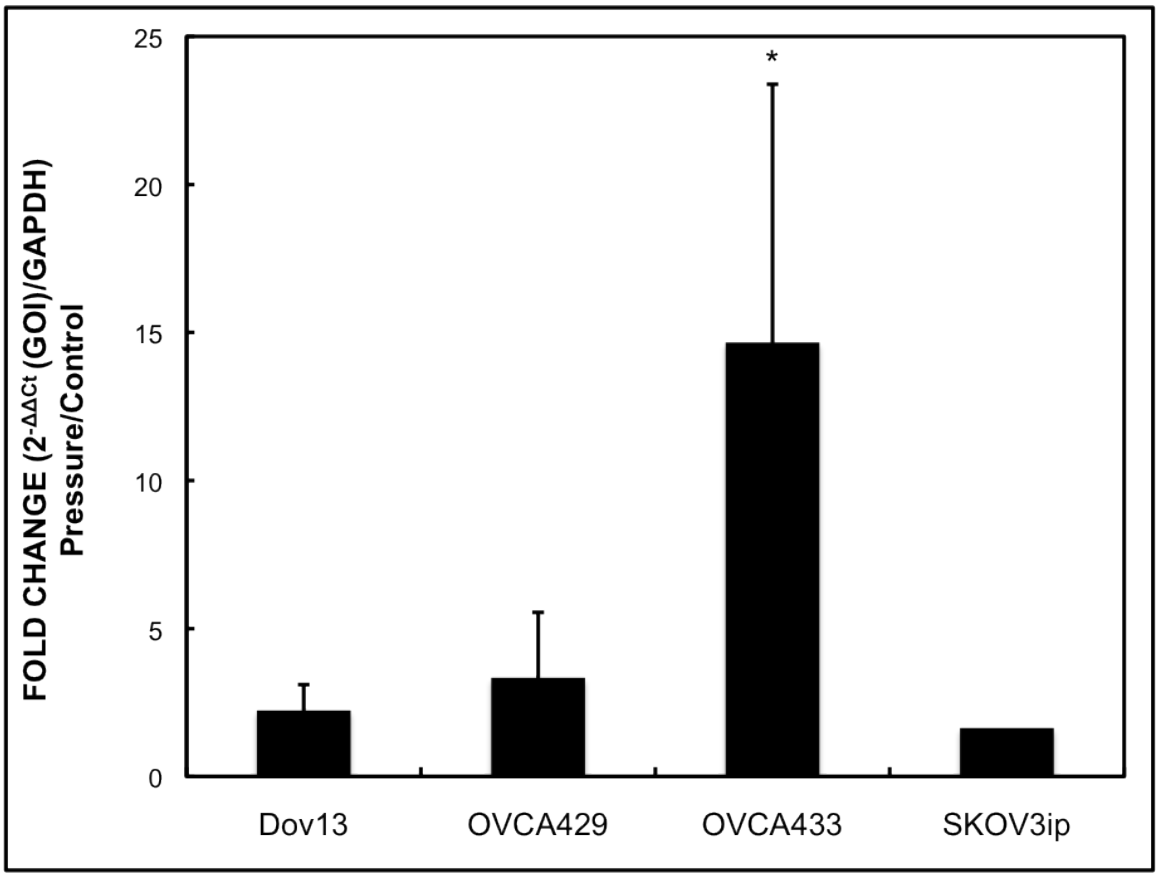


Figure 2.10: Increased Fluid Pressure-Cultured Cells Express *WNT5A* mRNA. Following 8-hour pressure culture at 25 mmHg (control: atmospheric pressure), isolation of RNA in TriZol and evaluation of *WNT5A* gene expression by real time RT-PCR was performed as described in Experimental Methods. *WNT5A* mRNA expression is increased (OVCA429, 3-fold; OVCA433, ~15-fold; DOV13, 2-fold; SKOV3ip, 2-fold) following exposure to increased fluid pressure, compared with control cell populations. Results represent the mean of a minimum of three independent experiments, except SKOV3ip which was only repeated twice.

2.11 WNT5A IS EXPRESSED IN ASCITES FLUID OF OVARIAN CANCER PATIENTS

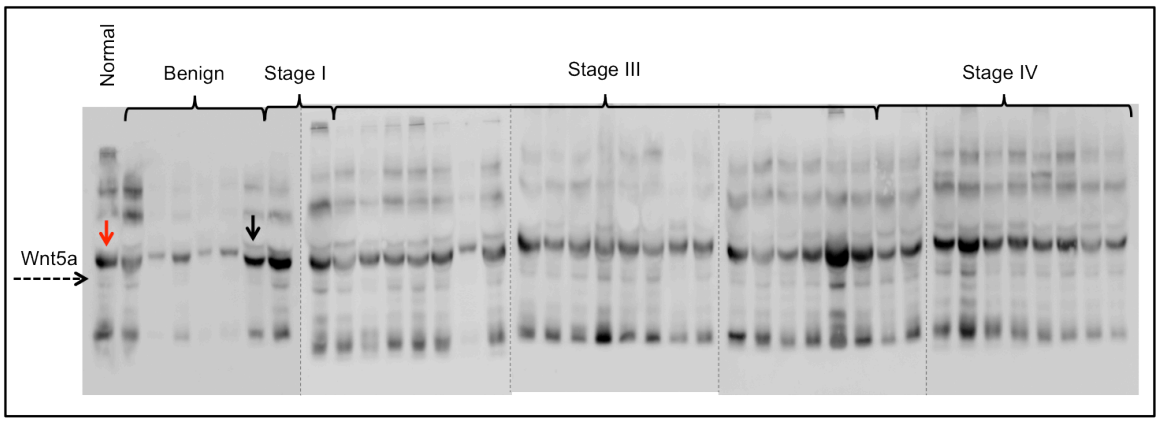


Figure 2.11: Wnt5a is Expressed in Ascites Fluid of Ovarian Cancer Patients. Ascites samples (20 µg total protein per lane) were evaluated for Wnt5a protein expression by gel electrophoresis (9%) and Western blot (anti-Wnt5a, R&D Systems, 1:1000) as described in Experimental Methods. Expression is higher in ovarian cancer patients' ascites, compared with benign ascites fluid and normal fluid.

2.12 WNT5A IS EXPRESSED IN ASCITES FLUID OF OVARIAN CANCER PATIENTS

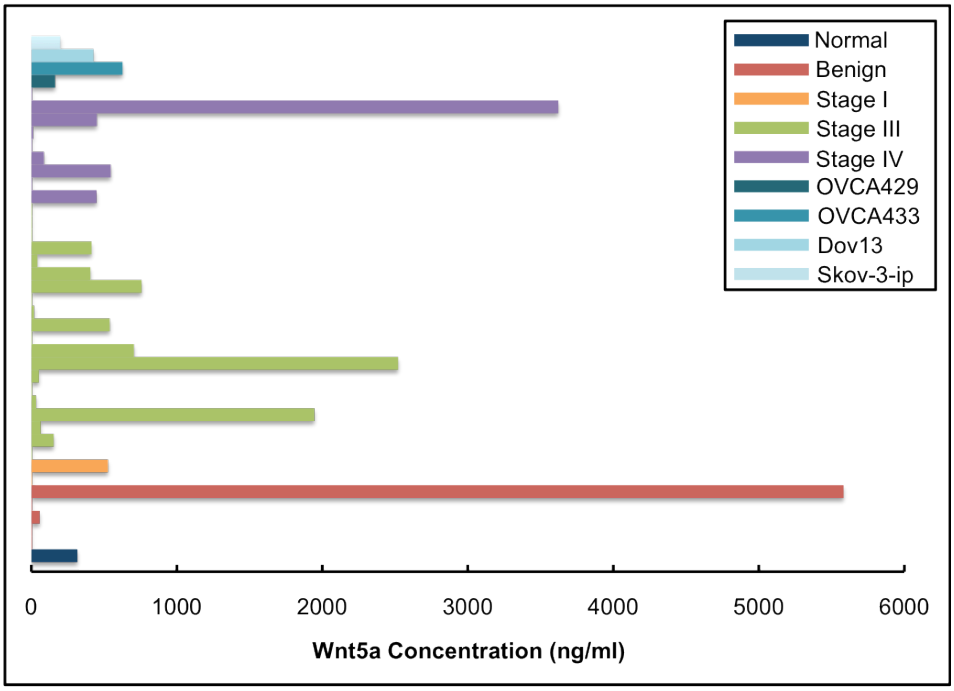


Figure 2.12: Wnt5a is Expressed in Ascites Fluid of Ovarian Cancer Patients. Wnt5a protein expression evaluated in ascites samples (50 µg total protein per well) by sandwich ELISA (USCNK Inc) following manufacturer's instructions. Wnt5a protein expression is higher in ovarian cancer patients' ascites, compared with benign ascites fluid and normal fluid.

2.1 WNT5A EXPRESSION IN ASCITES FLUID OF OVARIAN CANCER PATIENTS

	Normal	Benign	Stage I	Stage III	Stage IV
Wnt5a Conc. (ng/ml)	317	11.6*	263.5	402.7	516.8

Table 2.1: Wnt5a is Expressed in Ascites Fluid of Ovarian Cancer Patients. Wnt5a protein expression evaluated in ascites samples (50 µg) by sandwich ELISA (USCNK Inc). Average concentration of Wnt5a protein by FIGO stage (ng/ml) is shown. *Wnt5a value for expression in Ovarian Hyperstimulation Syndrome (OHSS) was omitted.

2.2 WNT5A EXPRESSION IN OVARIAN CARCINOMA CELL LINES

	OVCA429	OVCA433	DOV13	SKOV3ip	AVERAGE
Wnt5a Conc. (ng/ml)	164	627	428	199	354.5

Table 2.2: Wnt5a is Expressed in Ovarian Carcinoma Cell Lines. Wnt5a protein expression evaluated in ascites samples (50 µg) by sandwich ELISA (USCNK Inc).

2.13 WNT5A EXPRESSION MODULATES ADHESION IN OVARIAN CARCINOMA CELLS

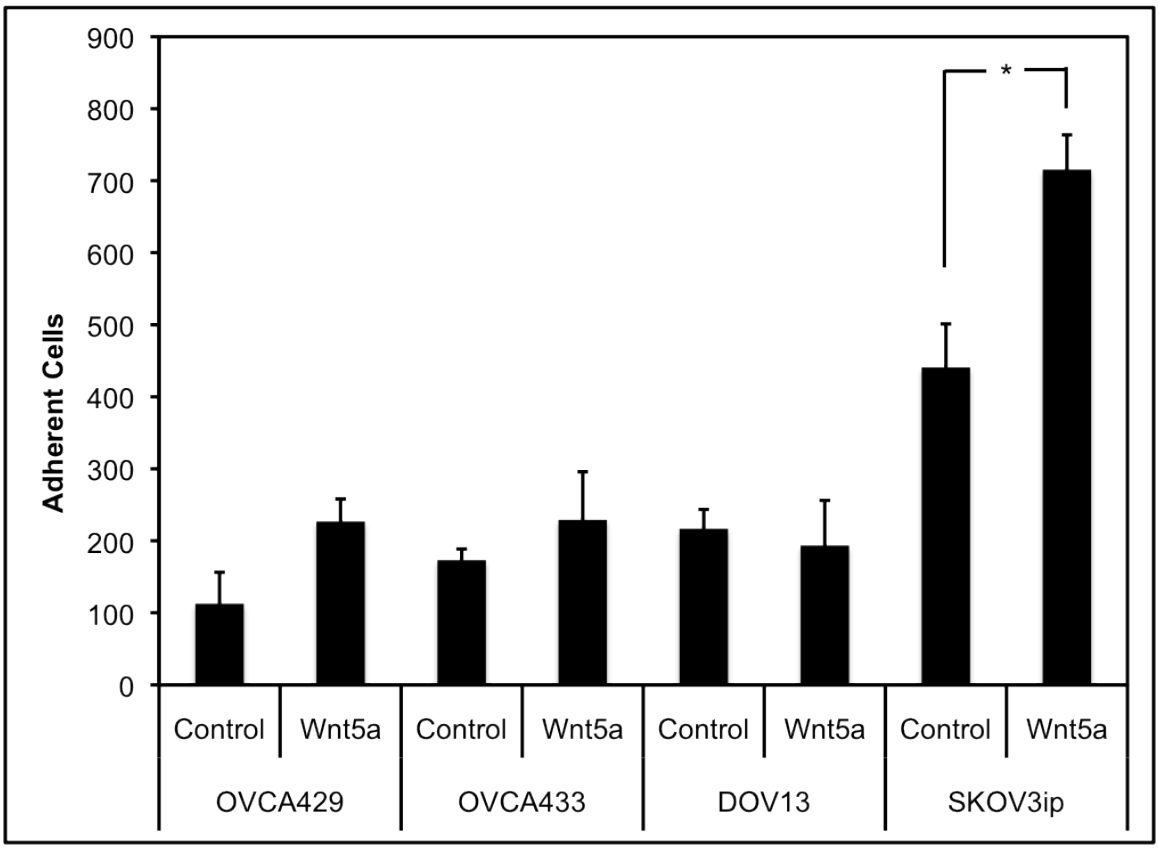


Figure 2.13: Wnt5a Expression Modulates Adhesion in Ovarian Carcinoma Cells. Cells were ectopically treated with Wnt5a (400 ng/ml) for 24 hours at 37°C, seeded onto 10 µg/ml collagen type I-coated culture dishes and allowed to adhere for 30 minutes (OVCA429, OVCA433) or 90 minutes (DOV13, SKOV3ip) at 37°C. SKOV3ip adhesion to Collagen I is increased over 50%; adhesion of OVCA429 and OVCA4233 is moderately increased (~20%).
*p<0.005

III. DISCUSSION

The influence of peritoneal mechanobiology distinguishes epithelial ovarian carcinoma (EOC) dissemination from other solid tumor metastatic cascades. EOC cells are shed from the surface of the ovary and float in accumulating ascites fluid, presenting unusual microenvironmental pressure dynamics. Although cellular modifications resulting from an altered mechano-environment are not well understood, this work provides a foundation for elucidating the functional consequences of biophysical modulation in ovarian cancer: increased intraperitoneal force modulates cell proliferation, cadherin dynamics, and Wnt/β-catenin gene expression in ovarian carcinoma. Further, the fluid pressure-upregulated Wnt signaling ligand Wnt5a is highly expressed in malignant ascites and may potentiate metastatic success via increased cell-matrix adhesion (**Fig. 2.14**).

EOC cell lines demonstrate altered proliferation patterns following culture under increased fluid pressure; OVCA429, OVCA433, and SKOV3ip cell proliferation is increased, whereas DOV13 cell proliferation is decreased following culture at 22

mmHg compared with atmospheric pressure-cultured controls. The data demonstrating increased proliferation in OVCA429, OVCA433, and SKOV3ip are consistent with several studies demonstrating increased proliferation in a human osteosarcoma cell line and a human chondrosarcoma cell line [Haskin et al, 1993; Takahashi et al, 1998]. Further, previous work investigating elevated tumor pressure in osteosarcoma, breast carcinoma and non-small cell lung tumor cell lines also found altered proliferation in cells cultured at 20 mmHg or 100 mmHg. Interestingly, the breast carcinoma cell line MCF7 exhibited higher proliferation following 72-hour culture at 20 mmHg, and lower proliferation at 100 mmHg [DiResta et al, 2005].

Protease activity is required for metastatic success in ovarian cancer. The matrix metalloproteases (MMPs) MMP-2, MMP-9, and MT1-MMP (MMP-14) are highly expressed by ovarian tumor cells, and found in ascites and peritoneal tissues. These proteases have also been shown to facilitate tumor metastasis via cleavage of cell-cell adhesion complexes (such as E-cadherin-based adherens junctions) and through extracellular matrix remodeling [di Nictolis et al, 1996; Ellerbroek et al, 1999; Stack et al, 1998]. uPA has also been shown to play an important role in ovarian tumor invasion, degrading extracellular matrix [Andreason et al, 2000] and uPA expression correlates inversely with poor prognosis [Schmafeldt et al, 1995; Kuhn et al, 1994]. Although uPA is unchanged following culture under increased fluid pressure, a number of genetic, epigenetic and microenvironmental factors may cumulatively contribute to

increased proteolytic activity in ovarian cancer. Future studies may also evaluate expression and proteolytic activity of the MMPs implicated in ovarian carcinoma.

A hallmark of tumor progression is loss of cell-cell adhesions, particularly E-cadherin homodimers [Liotta and Stetler Stevenson, 1991; Liotta 1992; Takeichi et al, 1991]. In ovarian cancer, there is an early gain of E-cadherin expression, as ovarian epithelium expresses N-cadherin, and a late loss of E-cadherin. Western blot evaluation of cell lines expressing either E-cadherin (OVCA429, OVCA433) or N-cadherin (DOV13, SKOV3ip) following increased fluid pressure reveals increased expression of endogenously expressed cadherins in all four cell lines. This observation is in contrast to expected results, but correlates with previous Stack laboratory observations that increased compressive force for 1 hour or 18 hours leads to increased E-cadherin expression. Interestingly, long-term (38 hours) compressive force leads to downregulation of E-cadherin [Symowicz and Stack, unpublished observations]. It is interesting to speculate that an early gain in E-cadherin expression reflects a compensatory mechanism by which cells seek to avoid apoptosis. In fact, E-cadherin-expressing cells have been shown to resist disaggregation during exposure to shear stress, compared with E-cadherin-negative cells [Byers et al, 1995]. This resistance to disaggregation, while inhibiting metastasis in other carcinoma tumor types, may protect disseminating ovarian multicellular aggregates in a novel fluid microenvironment. Although technical issues in the fluid-filled sac/pressurized pressure vessel currently prevent study of long-term pressure, it would be

interesting to evaluate cadherin dynamics over extended time periods. One method of circumventing the technical issues may be to confirm the short-term data using a Flexcell© and BioPress© culture plates. Unfortunately, this mechanism may complicate analysis of three-dimensional (3D) multicellular aggregates, which are a more pathophysiologically-relevant model than two-dimensional (2D) cell culture.

Emerging models and data have established the tenet that virtually all cells are capable of sensing change in the mechano-environment and initiating appropriate responses to maintain tensional homeostasis by converting biomechanical force into biochemical signals [Ingber, 1997; Ingber, 2003c; Ingber, 2008; Butcher et al, 2009]. Loss of tensional homeostasis can lead to not only phenotypic but also *genotypic* modifications that may potentiate tumor progression. Indeed, increased fluid pressure induced expression of *WNT5A* mRNA in OVCA429, OVCA433, DOV13 and SKOV3ip. *MMP9* and *SNAI1* (Snail1) mRNA expression are also modulated by culture under high fluid pressure. The mechanism(s) of this gene expression modulation is unclear; however, data support compression-induced β-catenin transcriptional activity in colon carcinoma [Whitehead et al, 2008]. Future studies will investigate the subcellular localization and transcriptional activity of β-catenin following culture under increased fluid pressure. It has been proposed that one mechanism for mechanically induced genetic alterations is coupling of extracellular matrix to the nucleus by cytoskeletal proteins, causing structural rearrangement of the

nucleus. In fact, a conserved complex known as the linker of nucleoskeleton and cytoskeleton (LINC), which physically couples nuclear matrix structure to the cytoskeletal network, has been identified [Crisp et al, 2006; Haque et al, 2006]. Although the exact structure of the nuclear matrix is unknown, it contains filament-like lamins, which play a central role in organizing transcriptional machinery and orienting chromosomes [Dechat et al, 2008; Lee et al, 2001; Worman et al, 1988]. Based on data confirming similar, although not identical, integrin clustering and activation in cells attached to extracellular matrix and mechanically-stimulated cells [Ingber, 1991], it may be postulated that similar nucleoskeleton rearrangements can be potentiated by both intact matrix and fluid-induced mechanostimulation. Taken together, these data implicate a role for mechanical regulation of nuclear structure and gene expression.

Expression of *WNT5A* mRNA in response to increased fluid pressure was unexpected, and the potential role for Wnt5a expression in ovarian carcinoma is unclear. Western blot and ELISA data reveal high expression of Wnt5a in ovarian tumor ascites and in EOC cell lines. However, Wnt5a expression was also strong in the peritoneal cavity in a normal patient and in a patient with ovarian hyperstimulation syndrome. The finding of Wnt5a expression in a normal patient may be explained by cycling levels of Wnt5a expression during the menstrual cycle [Punyadeera et al, 2005; Matsuoka et al, 2010]. Further, Wnt5a overexpression has been reported in a number of syndromes of the ovary [Jansen et al, 2004]. Although the data does suggest EOC cells as a source of

Wnt5a production (average concentration of 354.5 ng/ml), the sources for ascites-expressed Wnt5a are not clear. Wnt5a functions as a pro-inflammatory molecule in adipose tissue of obese human, and is highly expressed in these patients [Bilkovski et al, 2011; Schulte et al, 2012]. This is particularly interesting as obesity is negatively correlated with ovarian cancer prognosis [Olsen et al, 2007; Pavelka et al, 2006; Farifield et al, 2002]. Ectopic Wnt5a enhanced ovarian cancer cell line adhesion to Collagen type I. Physiologically, Wnt5a regulates convergent extension during embryogenesis, in part by mediating cell-matrix adhesion and cell migration [Wallingford et al, 2002]. Given its role in embryogenic cell migration and invasion, potential Wnt5a regulation of peritoneal invasion by EOC cells requires investigation.

Contradicting reports in the literature further complicate interpretation of the data. One report finds high expression of Wnt5a is a predictor of poor prognosis and a mediator of chemoresistance [Peng et al, 2011]. On the contrary, a second group finds high expression of Wnt5a suppresses EOC by promoting cellular senescence. One explanation for this apparent discrepancy is receptor context. Current dogma supports the theory that differential Wnt signaling is activated based on ligand context, with Wnt5a defined as a non-canonical, β-catenin-independent ligand [Hendrickx et al, 2008; Macdonald et al, 2007, Semenov et al, 2007]. Recent studies have challenged Wnt signaling dogma, suggesting that divergent Wnt pathway activation is Frizzled (Fzd) receptor and/or LRP co-receptor context-specific [Mikels and Nusse, 2006; van Amerongen et al, 2008].

Adding to the complexity of Wnt signaling, two non-Fzd Wnt receptors have been identified: h-Ryk and Ror2, both receptor tyrosine kinases [Katso et al, 1999; Kroiher et al, 2001; Xu and Nusse, 1998; Forrester et al, 2002; Oishi et al, 2007]. In fact, h-Ryk was originally isolated from the EOC cell line, SKOV3 [Wang et al, 1996] and is overexpressed in EOC [Wang et al, 1996; Katso et al, 1999; Katso et al, 2000]. The receptor context of tumor cell models used in this study remain unknown; elucidation of receptor expression may clarify the mechanisms of EOC response to increased Wnt5a expression.

Ascites-induced increases in intraperitoneal pressure have negative implications at the clinical and cellular levels. Development of tense ascites, usually in stage III and stage IV EOC is associated with severe discomfort, poor prognosis, and fatality in patients [Rahaman and Cohen, 2002; Puls et al, 1996]. The presence of ascites is correlated with higher intraperitoneal tumor spread [Ayhan et al, 2006]. Further, ovarian ascites-derived multicellular aggregates (MCAs) adhere to the extracellular matrix components fibronectin and Collagen type I [Burleson et al, 2004], which are both enriched in submesothelial matrix. Although the role of ascitic soluble factors, such as lysophosphatidic acid, in ovarian carcinoma have been studied in depth [Mills et al, 1988; Mills et al, 1990; Xu et al, 1995a; Xu et al, 1995b; Westermann et al, 1997; Xu et al, 1998; Furui et al, 1999; Fang et al, 2002; Yu et al, 2008], understanding the role(s) of mechanobiology requires more scrutiny.

The fluid-filled sac is a simple model of the peritoneal cavity, and does not represent the complex nature of the ovarian tumor microenvironment. The mesothelium is a dynamic tissue, which may participate in paracrine signaling interactions with tumor cells [Sako et al, 2003; Wang et al, 2005]. Further, ascites accumulation is in part an inflammatory response; the roles of inflammatory cells and their signaling ligands are not addressed in this work [Smith and Jayson, 2003; Freedman et al, 2004]. It is likely that multiple factors act in concert to facilitate ovarian cancer dissemination, and further studies are necessary to evaluate these complex metastatic events.

2.14 CHAPTER 2 SUMMARY

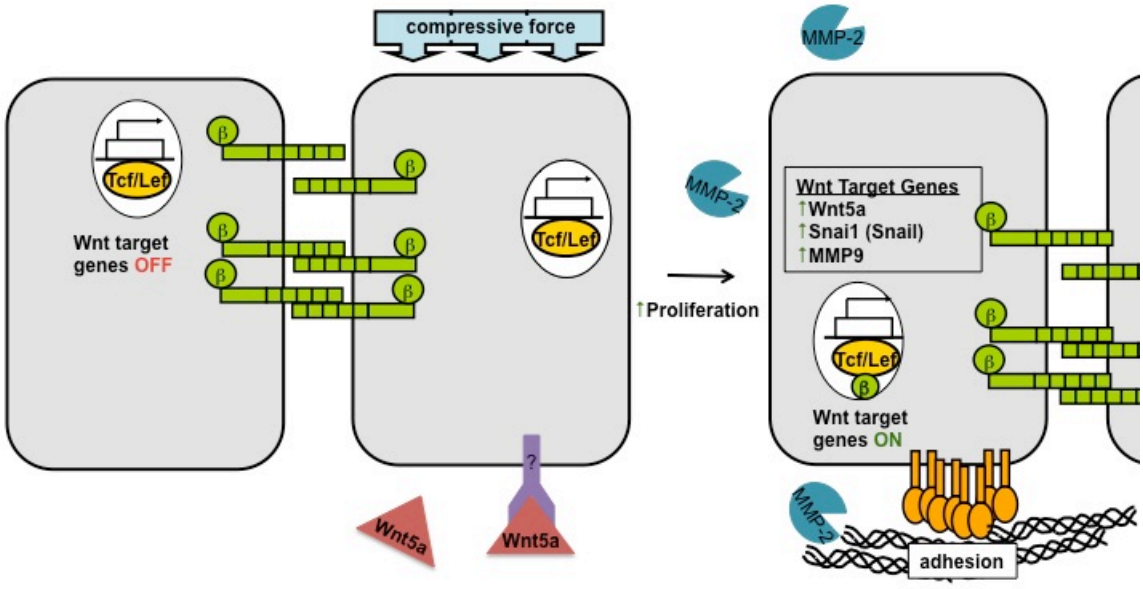


Figure 2.14: Chapter 2 Summary. Increased intraperitoneal fluid pressure modulates tumor cell proliferation, cell-cell adhesion and Wnt/β-catenin target gene expression. Ectopic expression of Wnt5a protein, a signaling ligand whose gene expression is upregulated by increased fluid pressure, is highly expressed in the ovarian tumor microenvironment and potentiates ovarian tumor cell adhesion.

3. LYSOPHOSPHATIDIC ACID INDUCES β-CATENIN-MEDIATED TRANSCRIPTION

I. RATIONALE

LPA, a bioactive lipid signaling molecule, plays a role in numerous cell processes including proliferation, migration, adhesion and cell survival [Moolenaar et al, 1992; Fishman et al, 2001] by acting at a family of G protein-coupled receptors (GPCRs) known as LPARs [Choi et al, 2010; Fukushima and Chun, 2001]. LPA has wide ranging influence on cell physiology and pathophysiology including increases in cytokine and growth factor expression, alteration of surface protein trafficking and modulation of transcription [Fang et al, 2000; Fang et al, 2004]. LPA, which is produced by both normal and malignant cells, is present in high concentration (2-80 μM) in ascites and serum from 98% of ovarian cancer patients, including those with early stage disease [Xu et al, 1995; Westermann et

al, 1998; Fang et al, 2002; Xiao et al, 2001; Mills et al, 2001; Xu et al, 2009; Xu et al, 1998]. Further, increasing LPA expression is correlated with poor prognosis, suggesting its potential role as a therapeutic biomarker [Xu et al, 1998]. Data from our laboratory and others demonstrates LPA regulation of proteases (MT1-MMP, MMP2, MMP9, uPA), inflammatory signaling molecules (Cox2, IL-8), and adhesion molecules including E-cadherin and β1 integrin [Do et al, 2007; Xu et al, 2009; Fishman et al, 2001; Gil et al, 2008; Symowicz et al, 2005].

LPA induces diverse cellular functions by activating one of five known receptors LPA_{1-5}, which modulate various signaling pathway proteins [Nelson et al, 2004; Yu et al, 2008; Meigs et al, 2001; Meigs et al, 2002; Yang et al, 2005; Fang et al, 2002]. LPA_1 (G_α12/13), LPA_3 (G_αq/11) and LPA_4 (G_α12/13) receptor subtypes are expressed in the ovary, with LPA_4 being the most abundant [Choi et al, 2010; Fukushima et al, 2001]. LPA2 and LPA3 are aberrantly over-expressed in several ovarian carcinoma cell lines [Fang et al, 2000; Fang et al, 2004]. Overexpression of LPA2 and/or LPA3 potentiates a more proliferative and invasive phenotype in ovarian tumor cells by modulating IL-6, IL-8 and VEGF expression [Yu et al, 2008]. Interestingly, heterotrimeric G proteins containing G_α12/13 interact with the cytoplasmic domain of E-cadherin, rescuing breast carcinoma cells from E-cadherin-mediated migration suppression, preventing E-cadherin-based cell aggregation, and displacing β-catenin from the adherens junction complex [Meigs et al, 2001; Meigs et al, 2002]. In colon cancer cell lines, LPA treatment (1μM) leads to robust inactivation of GSK3β and nuclear localization of β-catenin [Yang

et al, 2005]. These data correlate with a previous study demonstrating that LPA treatment (0.1-20 μM) or LPA_2/LPA_3 expression induced a deactivating phosphorylation of GSK-3β in HEK293 cells [Fang et al, 2002b]. Recently, it has been shown that the Gβγ subunit of the G protein heterotrimer can also mediate Wnt signaling, inhibiting β-catenin degradation and allowing β-catenin-mediated transcriptional activity [Jernigan et al, 2010]. Here, novel role(s) of LPA as a mediator of ovarian carcinoma progression through modulation of β-catenin-regulated gene expression are investigated.

II. RESULTS

Lysophosphatidic Acid Dissociates Epithelial Ovarian Carcinoma Monolayers.

Lysophosphatidic acid (LPA) is highly expressed in the ovarian tumor microenvironment (up to 80 μM [Westermann et al, 1998; Xu et al, 1995a, Xu et al, 1998; Xiao et al, 2001; Shen et al, 1998]), and previous work has shown LPA mediation of many cellular events in ovarian carcinomas [Erickson et al, 2001; Fang et al, 2002; Fishman et al, 2001; Liu et al, in press]. LPA concentration increases with tumor progression, peaking prior to and during establishment of metastases [Fang et al, 2000]. The transition from disseminating multicellular aggregates in ascites to cell adhesion and invasion at the metastatic site is facilitated by generating single cells via dissolution of E-cadherin-based cell-cell adhesions [Harisi et al, 2009]. To investigate the potential for LPA-mediated downregulation of junctional activity, two E-cadherin-expressing cell lines, OVCA429 and OVCA433, were cultured to 100% confluence with 40 μM LPA, 1% BSA in PBS as a control, for 24 hours. Intact monolayers were dissociated from the culture dish by dispase treatment, and monolayers were mechanically disrupted using a bench-top rotator for 50 rotations. Monolayer fragments,

regardless of size, were enumerated. Control-treated OVCA429 and OVCA433 cells maintained monolayer integrity, as shown in **Fig. 3.1A,C** and **Fig. 3.1B,E**, respectively. Treatment with 40 μM LPA disrupted monolayer integrity (OVCA429, **Fig. 3.1D**; OVCA433, **Fig. 3.1F**), resulting in a larger number of cellular fragments compared with control (OVCA429, **Fig 3.1A**; OVCA433, **Fig. 3.1B**).

Loss of Surface-Expressed β-catenin Expression in Ovarian Carcinoma.

Previous data has shown rapid dissolution of E-cadherin-based adherens junctions (AJs) in response to LPA. The fate of the AJ-stabilizing, transcriptional regulator, β-catenin, following loss of AJs is unclear. Evaluation of a human ovarian tumor tissue microarray containing 105 tumor tissue samples by immunohistochemistry demonstrates nuclear expression of β-catenin in each of the four EOC subtypes (**Fig. 3.2A-D, Table 3.1**). All of the epithelial ovarian carcinoma (EOC) histotypes examined in this tumor microarray demonstrated strong expression of nuclear β-catenin. Of 34 primary serous tumor samples, 50.0% were positive for nuclear β-catenin. Endometriod tumor samples also express nuclear β-catenin (84.6%). In mucinous and malignat mixed Müllerian tumor (MMMT) samples, 66.6% were nuclear β-catenin positive. Interestingly, there was also strong nuclear β-catenin staining in borderline tumors as well, with 78.9% exhibiting strong immunoreactivity. Of 8 primary clear cell EOC samples, 62.5% stained positive for nuclear β-catenin. Eight (7 serous, 1 clear cell)

metastatic (mets) lesions were also analyzed for nuclear β-catenin with 5 of the 7 serous exhibiting positive staining, in addition to positive immunoreactivity in the clear cell met (**Table 3.1**). These data demonstrate nuclear localization of β-catenin in EOC; however, the mechanism(s) of nuclear β-catenin accumulation are unclear.

In order to determine if LPA-induced adherens junction disruption leads to freed cytoplasmic/nuclear β-catenin, OVCA433 cells were treated with 40 μM LPA for 2, 4, or 6 hours and evaluated for β-catenin expression by immunofluorescent staining. Surface-expressed β-catenin is significantly decreased following 2-hour LPA treatment compared with control, which corresponds to loss of surface-expressed E-cadherin expression (**Fig. 3.3A-D**). Further, β-catenin perinuclear accumulation can be observed following two hour LPA treatment (**Fig. 3.1D**, *white arrows*). β-catenin surface expression is partially recovered at the 4- and 6-hour time points (**Fig. 3.3F,H**), whereas E-cadherin surface-expression continues to decrease **(Fig. 3.3E,G)**.

To determine whether LPA-mediated loss of β-catenin surface is a LPA receptor-dependent event, OVCA433 cells were either treated with LPA (40 μM) alone, pre-treated with the pharmacologic LPA receptor inhibitor, Ki16425 (40 μM, 30 minutes), or pre-treated and then treated with LPA. LPA treatment led to loss of β-catenin surface expression, and perinuclear (*white arrows*) and nuclear accumulation (*red arrows*) of β-catenin (**Fig. 3.4C**) compared with untreated

control (**Fig. 3.4A**), vehicle control (**Fig. 3.4B**), and Ki16425 alone (**Fig. 3.4D**). This loss of surface expression was rescued by pre-treatment with Ki16425 prior to LPA treatment (**Fig. 3.4E**). Similar to LPA-mediated loss of β-catenin surface expression, a GSK3-β (member of the β-catenin degradation complex) inhibitor also facilitates loss of surface-expressed β-catenin (positive control, **Fig. 3.4F**).

Lysophosphatidic Acid Potentiates Nuclear Accumulation of β-catenin.

Although the exact mechanism of β-catenin nuclear transport is unknown, cytoplasmic and perinuclear accumulation of β-catenin is coupled to increased nuclear expression. In order to evaluate nuclear β-catenin accumulation, OVCA429 and OVCA433 cells were either treated with LPA, pre-treated Ki16425, or both pre-treated with Ki16425 followed by LPA treatment as described above for two hours. Nuclear proteins were isolated by subcellular fractionation as described in Experimental Methods and evaluated by Western blot. Indeed, LPA treatment potentiates a 50% increase in nuclear β-catenin localization in both OVCA429 (**Fig. 3.5A**) and OVCA433 (**Fig. 3.5B**). Further, this nuclear accumulation following LPA treatment is abrogated by pre-treatment with the LPA receptor inhibitor, Ki16425, suggesting LPA receptor-dependent signaling is the mechanism regulating β-catenin relocalization (**Fig 3.5A,B**).

LPA-Induced Nuclear β-catenin Activates Transcription.

The β-catenin protein sequence does not contain a nuclear localization signal domain, and the mechanism of nuclear transport is unclear [Suh and Gumbiner, 2003; Krieghoff et al, 2006; Fagatto et al, 1998]. Therefore, it is important to validate transcriptional activity and gene expression regulation to demonstrate functional consequences of nuclear β-catenin. β-catenin activates transcription by displacing the transcriptional repressor Groucho in Tcf/Lef binding site-containing genes promoters (**Fig. 3.6A)**, and subsequently binding its' coactivators Tcf/Lef (and potentially other co-factors such as CBP) to initiate transcription (**Fig. 3.6B**). To determine interaction of nuclear β-catenin following LPA treatment, nuclear proteins from OVCA433 were isolated using a chromatin-based immunoprecipitation protocol as described in Experimental Methods. Nuclear proteins were incubated with protein A/G beads and 5 μg of anti-β-catenin antibody, and protein-protein binding of bead-captured β-catenin was evaluated by immunoblotting for Tcf. Tcf/β-catenin interaction was greater in cells treated with LPA, compared with controls. Pre-treatment with Ki16425 inhibited this co-immunoprecipitation, suggesting that LPA-mediated increases in Tcf/β-catenin are LPA receptor-dependent (**Fig. 3.7**). In order to measure activation of the β-catenin/Tcf/Lef transcriptional complex, OVCA429 and OVCA433 cells were transfected with a Renilla luciferase reporter construct and either a TOP (Tcf) reporter construct or a FOP (control) reporter construct then treated with LPA for 2, 8 or 24 hours. β-catenin/Tcf/Lef transcriptional activity is increased after 2-hour LPA treatment, and sustained at 8 and 30 hours in both OVCA429 and OVCA433 (**Fig. 3.8A,B**). Consequentially, LPA-mediated β-

catenin/Tcf/Lef transcriptional activity increase leads to upregulation of 5 β-catenin target genes: *VIM* (Vimentin), *WNT5A*, *LRP6*, *PTGS2* (Cox-2) and *SNAI1* (Snail1), as shown in **Fig. 3.9**.

3.1 LYSOPHOSPHATIDIC ACID DISRUPTS EPITHELIAL COHESION

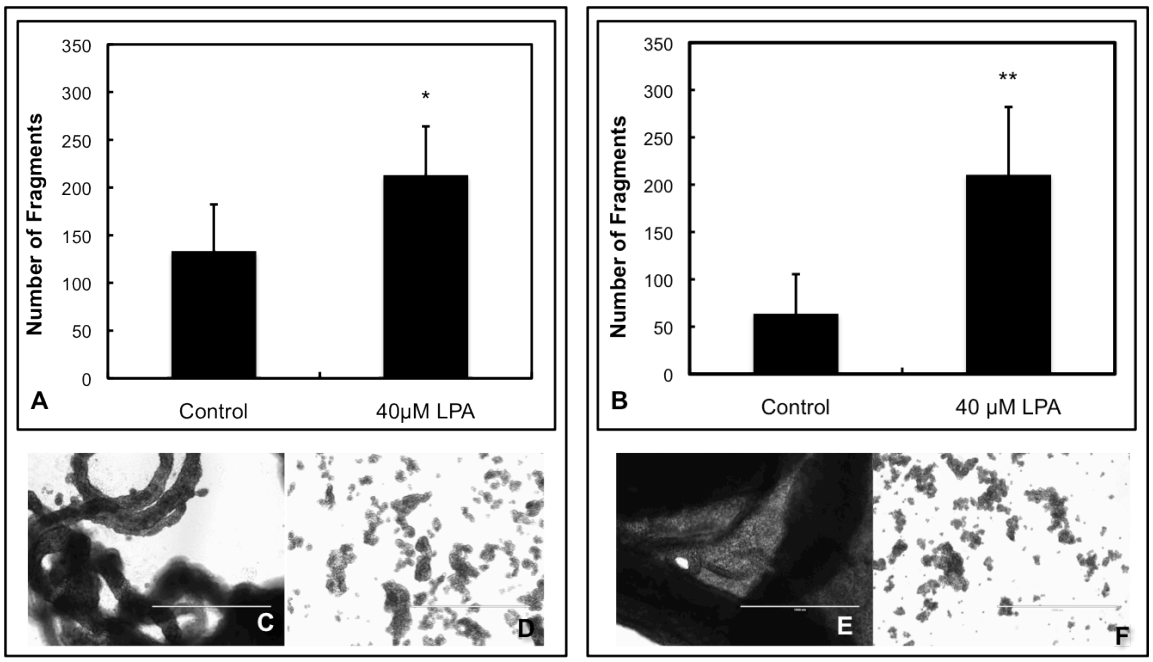


Figure 3.1: Lysophosphatidic Acid Disrupts Epithelial Cohesion. Confluent layers of (A) OVCA429 and (B) OVCA433 cells were treated with LPA (40 μM) for 24 hours. Cell sheets were detached using dispase (1 mg/ml in DMEM/F12 media), then subjected to 50 rotations on a bench-top rocker. Monolayer fragments were then enumerated, regardless of size. OVCA429 (C,D) and OVCA433 (E,F) cells demonstrate dispase-induced detachment from the culture dish as an intact sheet, but LPA-treated monolayers are fragmented (D,F) following mechanical agitation compared with control (C,E) cell.
*$p<0.02$, **$p<0.002$

3.2 β-CATENIN IS EXPRESSED IN HUMAN OVARIAN CARCINOMA

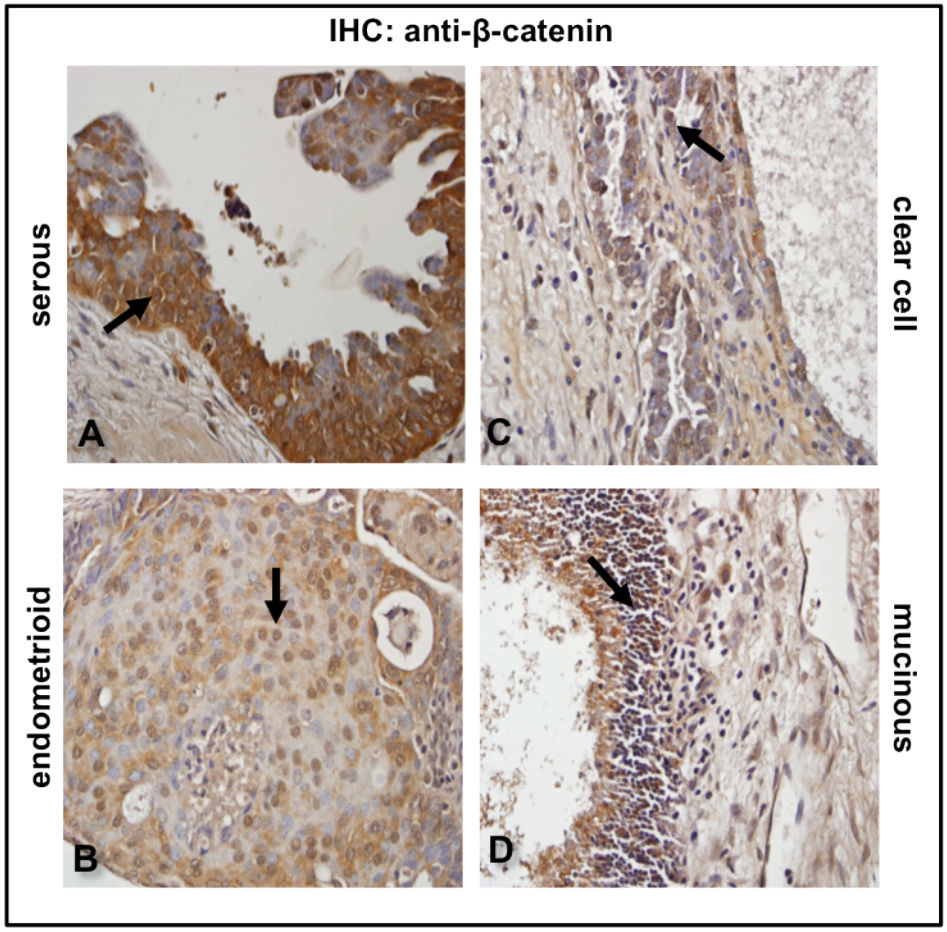


Figure 3.2: β-catenin Is Expressed in Human Ovarian Carcinoma. Representative samples of each of the four EOC subtypes were stained by immunohistochemistry with anti-β-catenin (BD Transduction Labs, 1:50). Nuclear β-catenin, indicated by *black arrows*, is found in serous (*A*), endometrioid (*B*), clear cell (*C*), and mucinous (*D*) tumors, with the heaviest staining found in serous subtype.

3.1 β-CATENIN EXPRESSION IN HUMAN OVARIAN CARCINOMA

	Number	Nuclear β -catenin
Serous		
Primary	34	17 (50.0%)
Metastatic	7	5 (71.4%)
Endometrioid		
Primary	26	22 (84.6%)
Metastatic	-	-
MMMT		
Primary	3	2 (66.6%)
Metastatic	-	-
Mucinous		
Primary	6	4 (66.6%)
Metastatic	-	-
Clear Cell		
Primary	8	5 (62.5%)
Metastatic	1	1 (100.0%)
Borderline		
Primary	19	15 (78.9%)
Metastatic	-	-
Untyped		
Primary	1	0 (0.0%)
Metastatic	-	-

Table 3.1: β-catenin Expression in Human Ovarian Carcinoma. A tissue microarray containing 105 tumor samples (3-5 μM thick, 1 mm diameter) was stained by immunohistochemistry, following antigen retrieval at 99°C in 10 mM Tris and 1 mM EDTA, pH 9.0, with anti-β-catenin (BD Transduction Labs, 1:50). Over 67% of all samples were positive for nuclear β-catenin expression, including 75% positive nuclear β-catenin staining in metastases.

3.3 LPA MEDIATES LOSS OF E-CADHERIN AND β-CATENIN SURFACE EXPRESSION

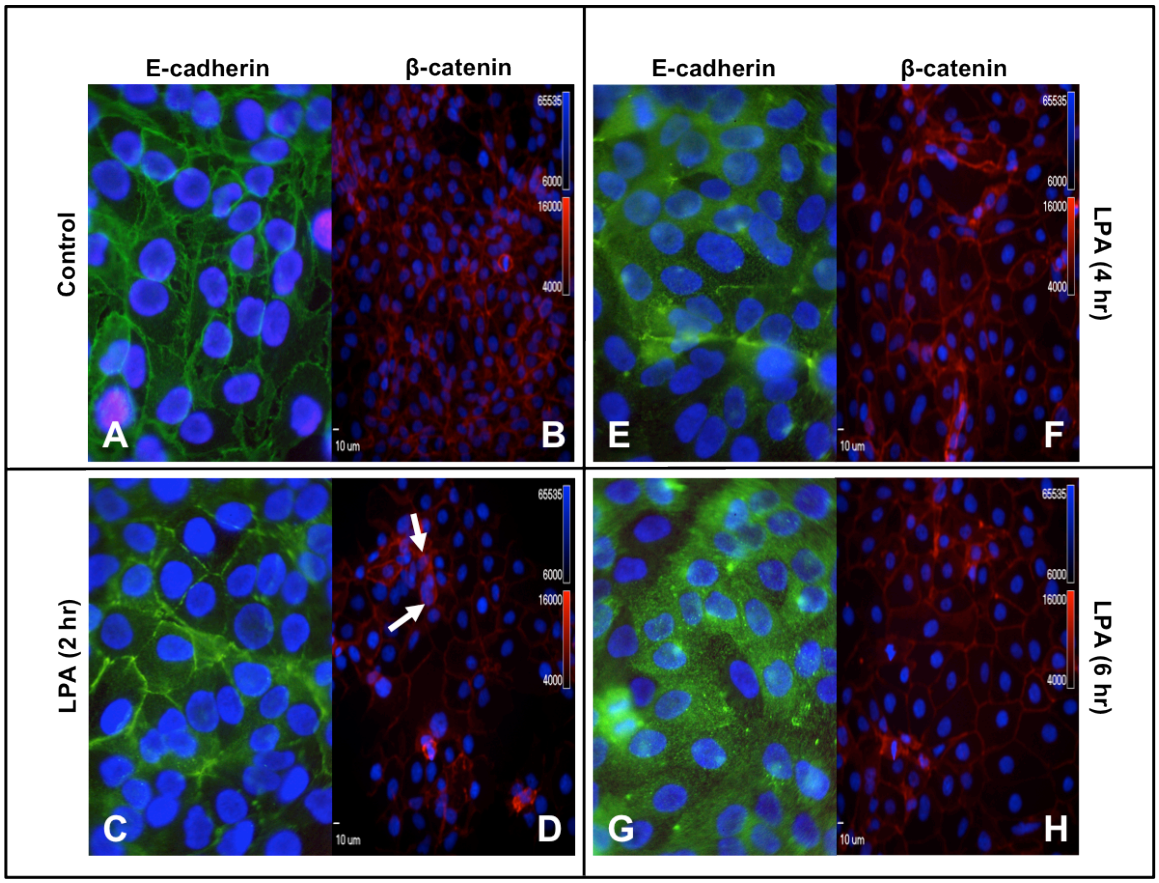


Figure 3.3: LPA Mediates Loss of E-cadherin and β-catenin Surface Expression. Serum-starved OVCA433 cells were untreated (A,B) or treated with 40 μM LPA for 2 hours (C,D), 4 hours (E,F), 6 hours (G,H), fixed in 4% paraformaldehyde, permeabilized in 0.3% Triton (anti-β-catenin slides only), and then stained for expression of E-cadherin (A,C,E,G; anti-E-cadherin, 1:300, *green*) or β-catenin (B,D,F,H; anti-β-catenin, 1:100, *red*). Surface expression of E-cadherin is lost after 2 hour LPA treatment, and sustained through 6 hour treatment. Surface-expressed β-catenin is decreased following 2 hour LPA treatment, and nuclear localization is observed (*white arrows*). β-catenin surface expression recovers at 4 and 6 hours.

3.4 LPA MEDIATES LOSS OF SURFACE-EXPRESSED β-CATENIN IN A LPA RECEPTOR-DEPENDENT MANNER

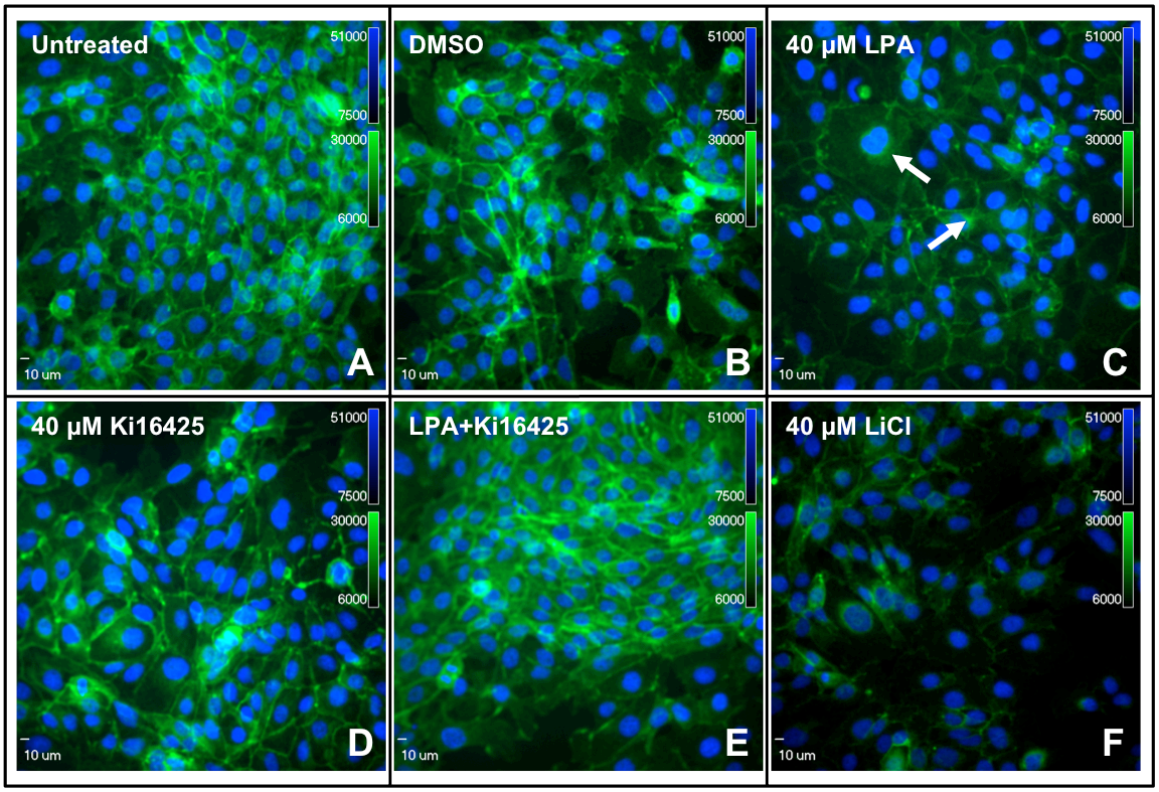


Figure 3.4: LPA Mediates Loss of Surface-Expressed β-catenin in a LPA Receptor-Dependent Manner. Cells were serum-starved overnight then pre-treated where appropriate with the LPA receptor inhibitor, Ki16425 (40 μM; D,E) or DMSO (B). Cells were then treated with 40 μM LPA (C,E) for 2 hours. Controls included untreated cells (negative control; A) and cells treated with the GSK3-β inhibitor, 40 μM LiCL (positive control; F). Cells were fixed in 4% paraformaldehyde, permeabilized in 0.3% Triton and immunofluorescently stained (primary: anti-β-catenin, 1:200; secondary: Alexa fluor 488-conjugated mouse anti-IgG, 1:500). LPA-treated cells exhibited decreased surface expression of β-catenin (green), and increased perinuclear β-catenin staining (C; *white arrows*). This decrease in β-catenin staining was abrogated by pre-treatment with Ki16425 (E).

3.5 LPA INDUCES LPA RECEPTOR-DEPENDENT NUCLEAR TRANSLOCATION OF β-CATENIN

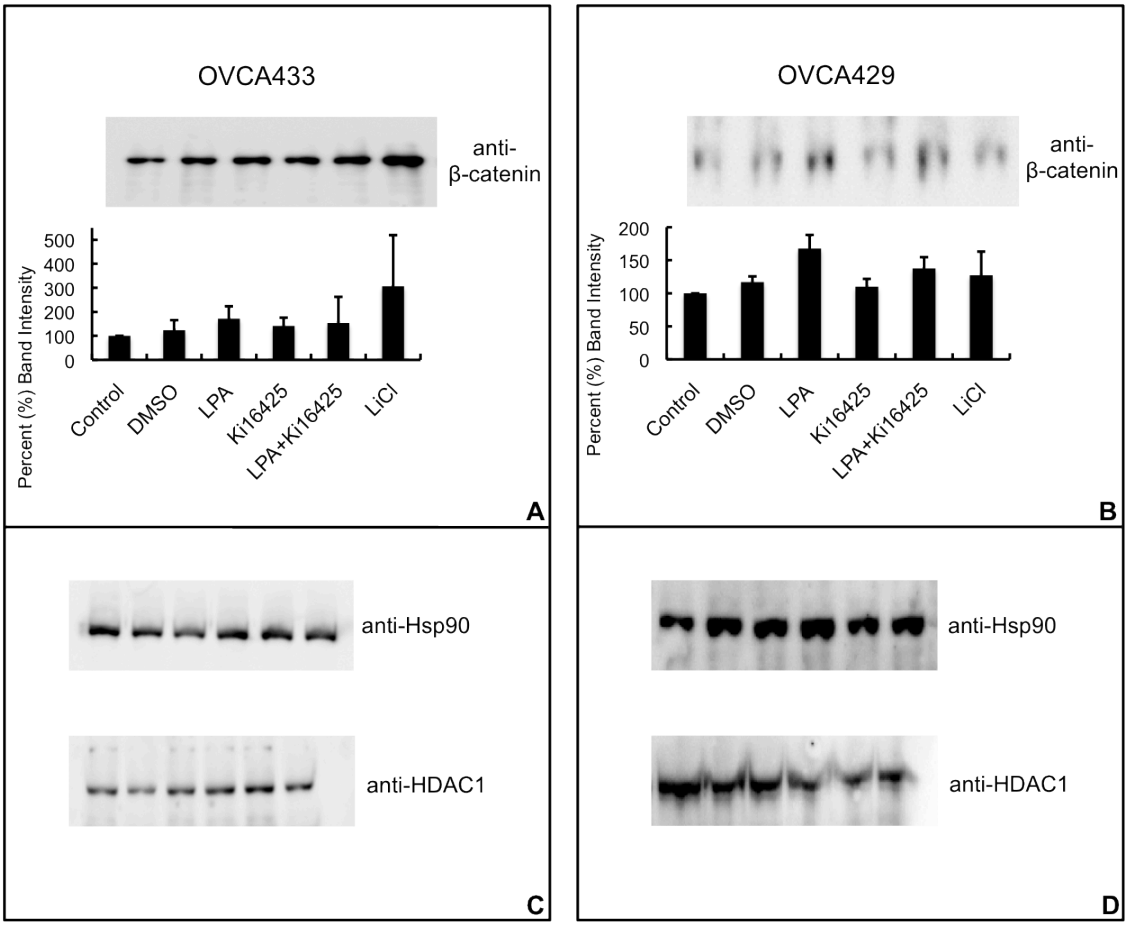


FIGURE 3.5: LPA Induces LPA Receptor-Dependent Nuclear Translocation of β-catenin. Cells were treated as labeled (LPA, 40 μM; Ki16425, LPA receptor inhibitor, 40μM; LiCl, positive control, 40 μM), then subjected to subcellular fractionation as described in Experimental Methods. Cell lysates were collected in mRIPA buffer, run on SDS-PAGE electrophoresis gels, and immunoblotted for β-catenin expression (anti-β-catenin, 1:1000). β-catenin staining in the nuclear fraction of both OVCA433 (A) and OVCA429 (B) cell lines was increased approximately 50% in LPA-treated cells compared with control. Inhibition of the LPA-LPA receptor interaction blocks this nuclear translocation. Fractionation efficiency was confirmed by washing (buffer: 50 mM Tris, pH 6.8, 1% SDS, 150 mM NaCl, 100 mM β-mercaptoethanol, 0.02% sodium azide) anti-β-catenin antibody off the membrane and re-probing for Hsp90, as a cytoplasmic marker, and HDAC1, as a nuclear marker, in both OVCA429 and OVCA433 lysates (C,D).

3.6 β-CATENIN AS A TRANSCRIPTION FACTOR

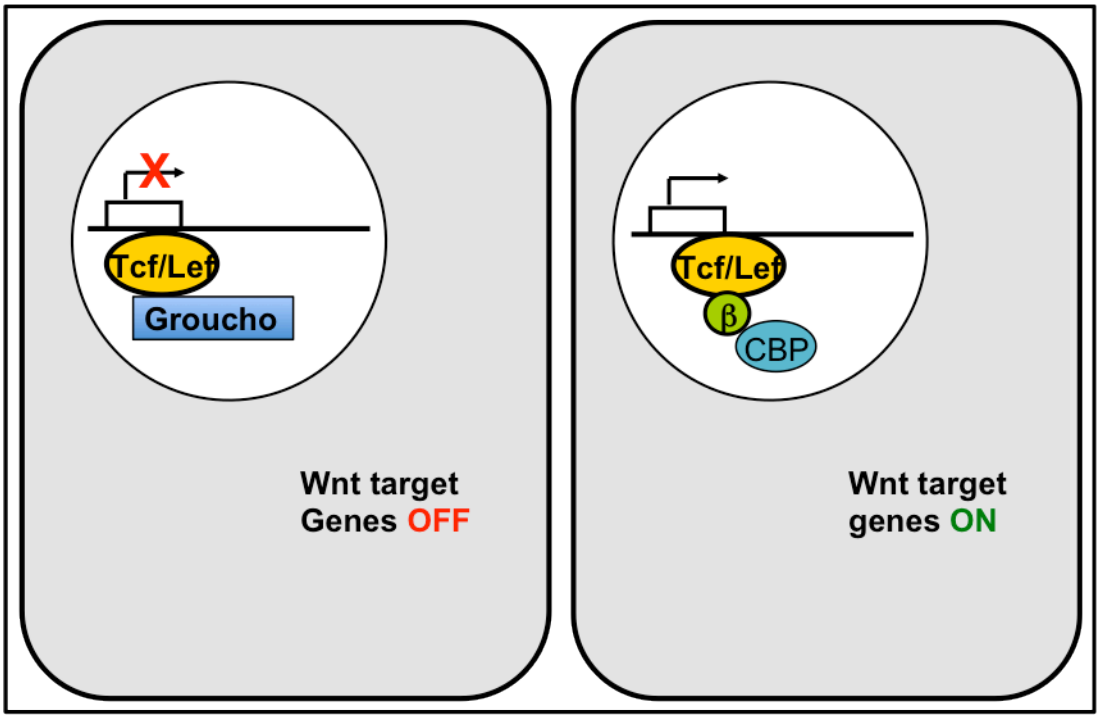


Figure 3.6: β-catenin as a Transcription Factor. After localizing in the nucleus, β-catenin displaces Groucho and binds the Tcf/Lef protein complex, activating transcription of genes whose promoters contain Tcf/Lef binding sites. Additional accessory proteins, such as CBP, may be recruited to the complex to faciliate transcription.

3.7 β-CATENIN COLOCALIZES WITH TCF FOLLOWING LPA TREATMENT

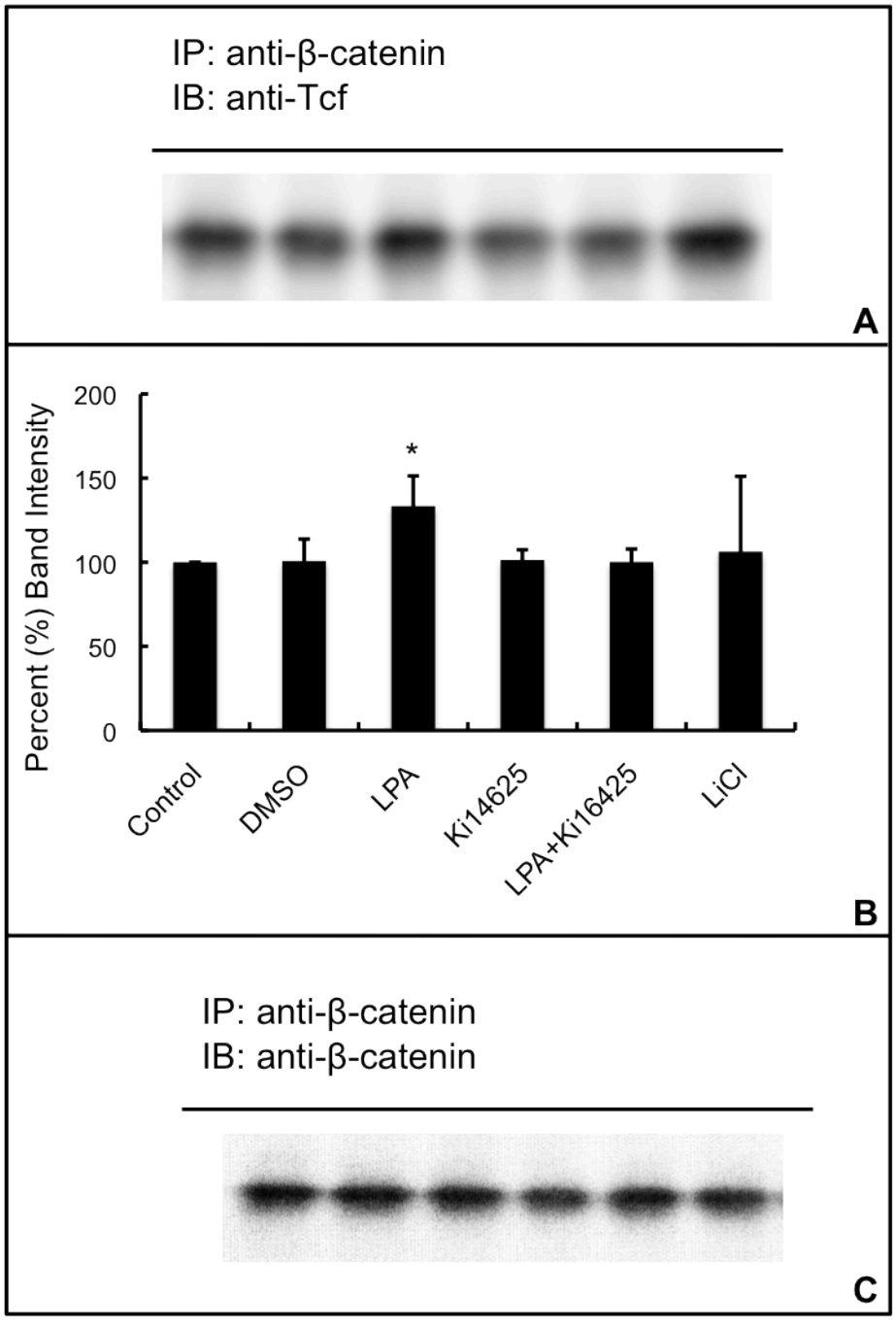
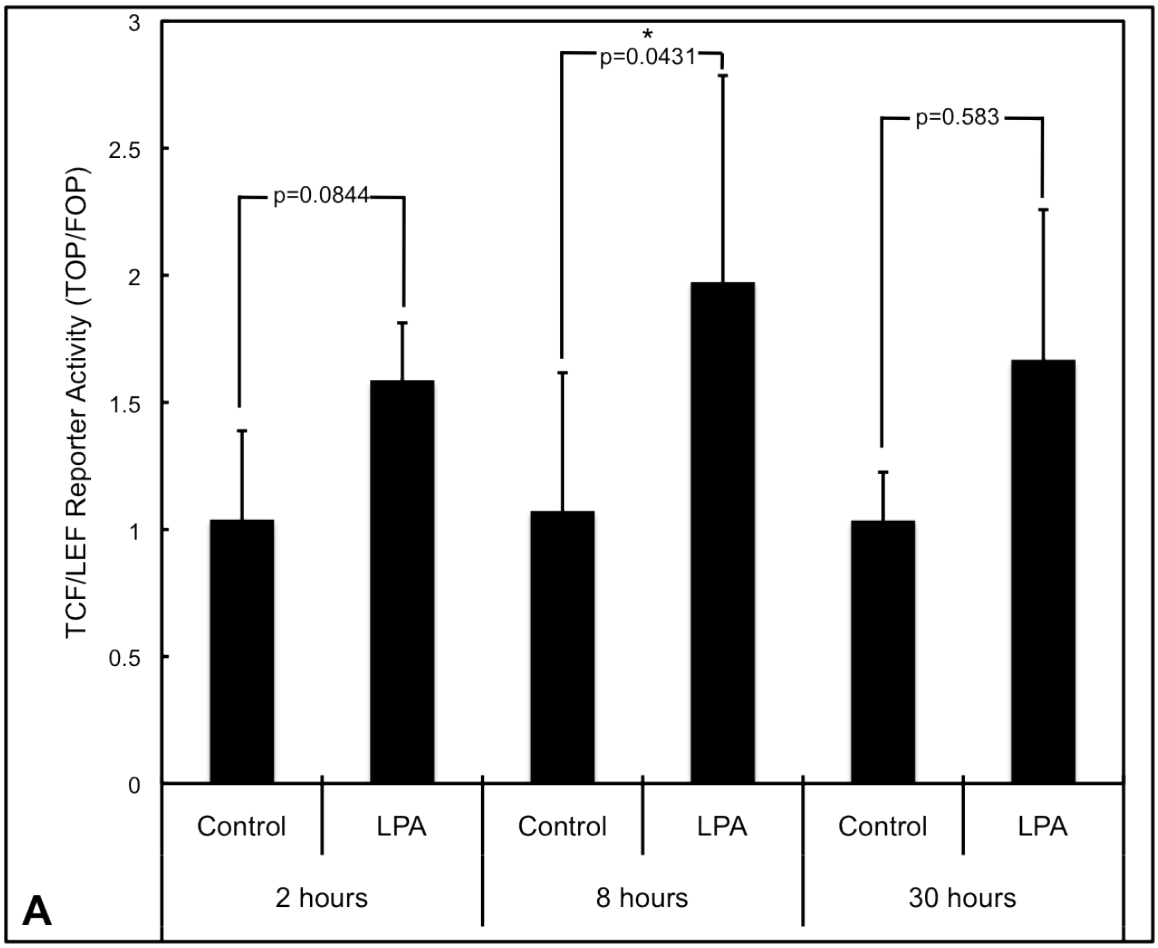


FIGURE 3.7: β-catenin Colocalizes with Tcf Following LPA Treatment. Cells were treated as labeled (LPA-40μM, Ki16425, LPA receptor inhibitor,-40μM, LiCl, GSK3-β inhibitor/positive control-40μM). Nuclear proteins were purified as described in Experimental Methods, and incubated with protein A/G beads and 5 μg anti-β-catenin antibody overnight. Beads were washed, and bound proteins were released by boiling in sample buffer. Samples were analyzed by SDS-PAGE gel electrophoresis and immunoblotted with an anti-Tcf antibody (Kamiya, 1:1000). A) Colocalization of β-catenin with Tcf is increased in response to 2 hour LPA treatment. B) Band intensity was quantified using FUJIFILM Multigauge V3.0 and is represented as percent of control. C) Anti-Tcf antibody was removed from PVDF membrane by washing in stripping buffer (50 mM Tris, pH 6.8, 1% SDS, 150 mM NaCl, 100 mM β-mercaptoethanol, 0.02% sodium azide) for 5 minutes, and membrane was re-probed with anti-β-catenin (1:1000) as a loading control.
*p<0.05

3.8 LPA-MEDIATED ACTIVATION OF THE TCF/LEF REPORTER IN OVARIAN CARCINOMA CELL LINES



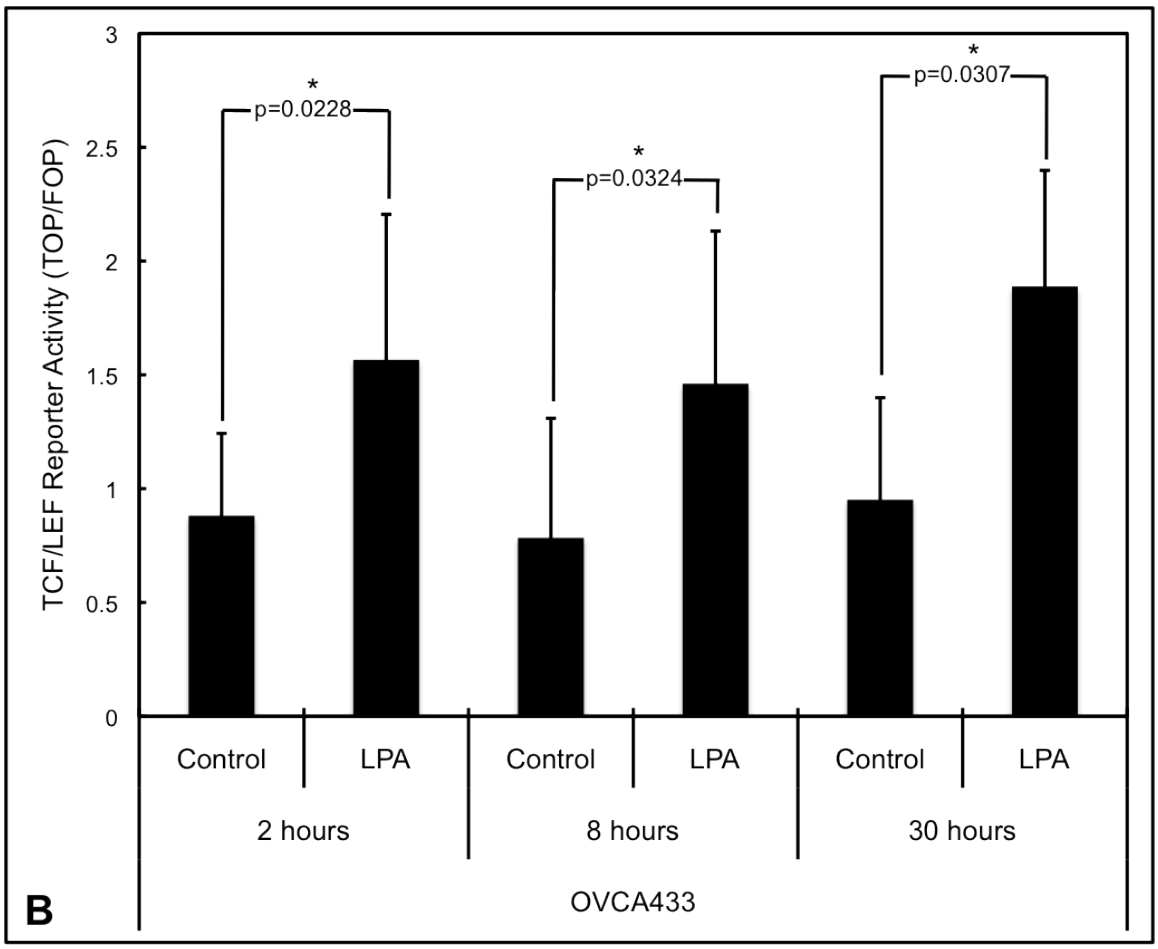


FIGURE 3.8: LPA-Mediated Activation of the Tcf/Lef Reporter in Ovarian Carcinoma Cell Lines. Cells were transiently co-transfected with either FOP reporter construct/Renilla luciferase reporter construct or TOP reporter construct/Renilla luciferase reporter construct, then treated with 40 μM LPA for 2, 8, or 30 hours as indicated. Luciferase reporter activity was measured using luminometer as described in Experimental Methods. Tcf/Lef reporter activity was increased in response to 2 hour LPA treatment, and sustained at 8 hours and 30 hours in both OVCA429 (A) and OVCA433 (B) cell lines. *p<0.05

3.9 LPA INDUCES TRANSCRIPTION OF β-CATENIN TARGET GENES

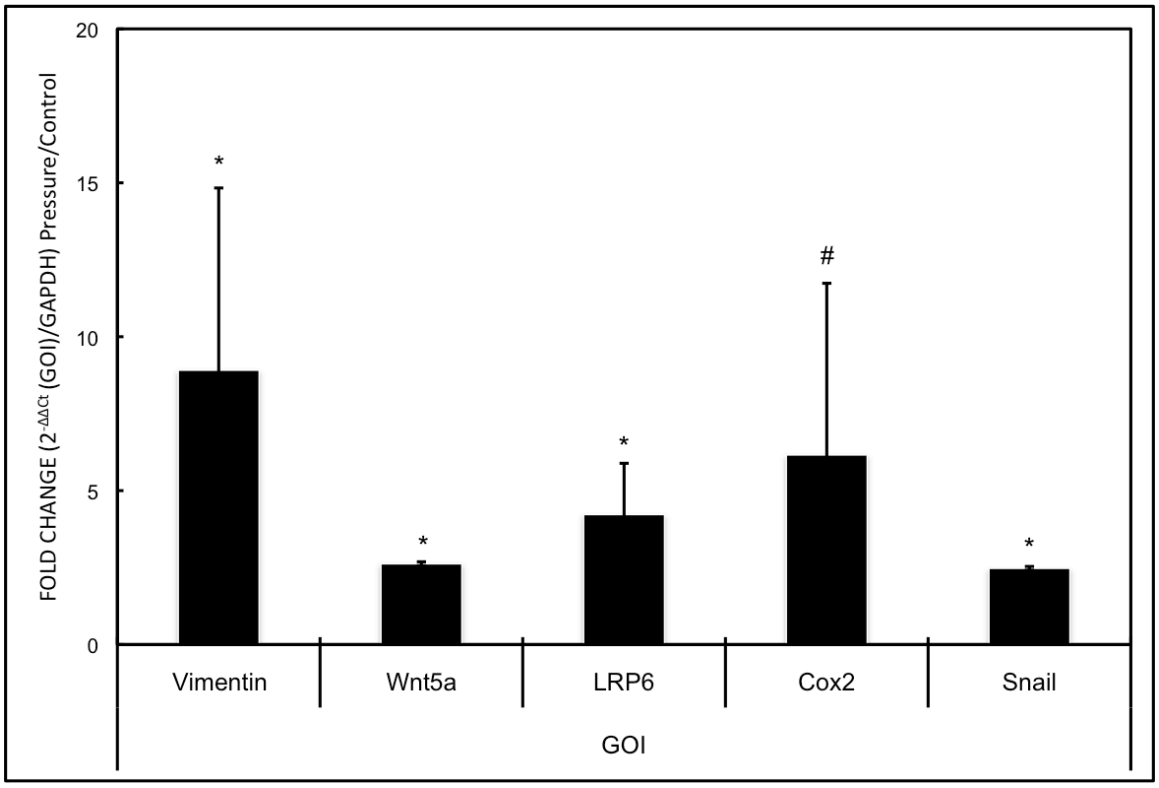


Figure 3.9: LPA Induces Transcription of β-catenin Target Genes. OVCA433 cells were treated with 40 μM LPA, or 1% BSA in PBS control, and total RNA was isolated and analyzed for changes in gene expression by RT-PCR. Data represents the mean of 4 independent experiments.
*p<0.05, #p<0.1

III. DISCUSSION

Lysophosphatidic acid (LPA) is highly expressed in the ovarian cancer microenvironment, and regulates a multitude of ovarian tumor cell responses including proliferation, epithelial-to-mesenchymal transition, migration and invasion. LPA is expressed as high as 80 μM in the ascites fluid and serum of patients with ovarian cancer [Westermann et al, 1998; Xu et al, 1995a, Xu et al, 1998; Xiao et al, 2001; Shen et al, 1998], underlining the importance of understanding its pathophysiological role in ovarian cancer. Data in this chapter define a novel role for LPA, ligand-independent activation of β-catenin-regulated transcription in ovarian carcinoma.

Treatment of intact OVCA429 and OVCA433 monolayers with LPA leads to fragmenting of the monolayer. This data demonstrates LPA-modulated loss of epithelial cohesion, as a functional result of disrupting cell-cell junctions. LPA is known to disperse EOC cell colonies [Jourquin et al, 2006], and to disrupt E-cadherin-based adherens junctions through protease-dependent cleavage and altered cadherin trafficking in ovarian carcinoma [Liu et al, in press]. The fate of β-catenin in response to LPA-mediated AJ dissolution, however, had not been

previously studied. Data presented here demonstrate that although loss of plasma membrane-localized E-cadherin is sustained, β-catenin loss from the cell surface appears to be a transient event, peaking after two hours. It remains unclear whether recycled β-catenin is reassociated with cell-cell junctions after 4 and 6 hours of LPA treatment, and if so what transmembrane protein forms these junctions. Interestingly, freed β-catenin accumulation is observed just around the nucleus in a perinuclear space following LPA treatment in ovarian carcinoma cells. This observation corresponds with data that first identified an intersection between adhesion-related and transcription-related cytoplasmic pools of β-catenin [Kam and Quaranta, 2009]. The theory that cytoplasmic β-catenin pools intersect nuclear β-catenin pools is further supported by data demonstrating increased nuclear localization, Tcf/Lef reporter activity and β-catenin target gene transcription in response to LPA treatment.

Current data identifies five pro-metastatic β-catenin target genes upregulated following LPA treatment: *VIM* (Vimentin), *WNT5A*, *LRP6*, *PTGS2* (Cox-2), and *SNAI1* (Snail1). Cox-2 contributes to tumorigenesis by inhibiting apoptosis, increasing growth factor expression to promote angiogenesis, and by enhancing matrix metalloproteinase (MMP) expression to stimulate invasion [Dempke et al, 2001]. Cox-2 protein is expressed in ovarian carcinoma, and functions as a downstream effector of LPA-mediated ovarian tumor cell migration and invasion. [Symowicz et al, 2005]. The proteinase MMP-9 is important for matrix invasion, is elevated in invasive ovarian cancer specimens as well as ovarian carcinomatous

ascites, and is correlated with lymph node metastasis [Young et al, 1996; Huang et al, 2000; Sakata et al, 2000]. In addition to enzymes and enzyme receptors expression of two genes commonly associated with EMT, vimentin and snail, was also induced by LPA treatment. Ovarian cancers typically display both epithelial and mesenchymal characteristics and the mesenchymal marker vimentin is widely expressed in tumor specimens [Hudson et al, 2008]. Snail is a key inducer of EMT and functions as a negative regulator of E-cadherin transcription. Several studies have demonstrated that nuclear localization of Snail correlates with tumor progression, with enhanced Snail immunoreactivity in metastatic lesions. [Tuhkanen et al, 2009; Jin et al, 2010]. Furthermore, patients with both primary and metastatic tumors positive for Snail expression showed a significant decrease in overall survival [Blechschmidt et al, 2008]. LRP6 functions as a Wnt co-receptor that recruits Axin and Dishevelled to the plasma membrane, thereby disrupting the degradation of β-catenin and facilitating β-catenin nuclear translocation. LRP6 is expressed by ovarian carcinoma cell lines (current data and unpublished observations); however, expression in ovarian tumor tissues has not been evaluated. It is important to note that while LPA-induced loss of β-catenin (2 hour treatment) is recovered after 4 hours and 6 hours, Tcf/Lef reporter activity remains increased for up to 30 hours. This discrepancy may be secondary to the regulation of nuclear transport or the “half-life” of β-catenin in the nucleus; however, speculation is difficult since the mechanism of β-catenin transport remains a topic of debate.

The exact mechanism by which LPA signaling is conferred in EOC is unclear. It cannot be overlooked that chronic exposure of disseminating EOC cells to high (up to 80 μM) environmental LPA may lead to desensitization via downregulation of LPA receptor surface expression, a regulation phenomenon common to G-protein-coupled receptors [Lefkowitz, 1993]. Under these conditions, LPA may confer observed changes in cell phenotype and/or genotype in a receptor-independent manner. Early evidence for receptor-independent LPA signaling includes observations that invertebrates produce and are responsive to LPA in the absence of conservation of LPA-encoding genes [Saba, 2004]. Further, inhibition of LPA receptors in platelets does not abrogate LPA-mediated mitogenesis and platelet aggregation, suggesting a low-affinity, receptor-independent LPA signaling pathway in these cells [Hooks et al, 2001]. Direct membrane perturbation has been proposed as a potential receptor-independent mechanism of LPA due to its detergent-like structure [Fukushima et al, 2002]. Neurobiology studies have shown that LPA is cleaved to PA by endophilin I, and that PA can mediate endocytosis at the neural synapse [Schmidt et al, 1999]. Although evidence for LPA signaling independent of the LPA receptors exists, further studies are necessary to elucidate specific mechanisms of LPA signaling in ovarian carcinomas.

Downstream of receptor-dependent and/or receptor-independent activation, the LPA-mediated mechanisms that regulate β-catenin localization and subsequent signaling are unknown. It could be assumed that a rapid increase in cytoplasmic

pools of β-catenin saturates degradation complexes allowing for nuclear translocation, but this claim cannot currently be substantiated. LPA transactivates a number of receptors, suggesting a mechanism for indirect regulation of β-catenin localization. Data has shown an interaction between cadherin cytoplasmic tails and the heterotrimer G protein, $G\alpha_{12}$ [Meigs et al, 2001]. These data are of particular interest since the ovarian cancer-related LPA receptors ($LPAR_2$ and $LPAR_3$) are $G\alpha_{12/13}$-type G-proteins. Activation of the G protein displaces β-catenin from the cadherin, potentially activating transcriptional pathways [Meigs et al, 2001]. Regardless of the mechanism, the aforementioned data demonstrates activation of Wnt/β-catenin transcription activity following LPA-mediated loss of adherens junctions, and adds to the body of literature supporting a nexus between the LPA and cadherin/catenin signaling cascades (**Fig. 3.9**).

3.9 CHAPTER 3 SUMMARY

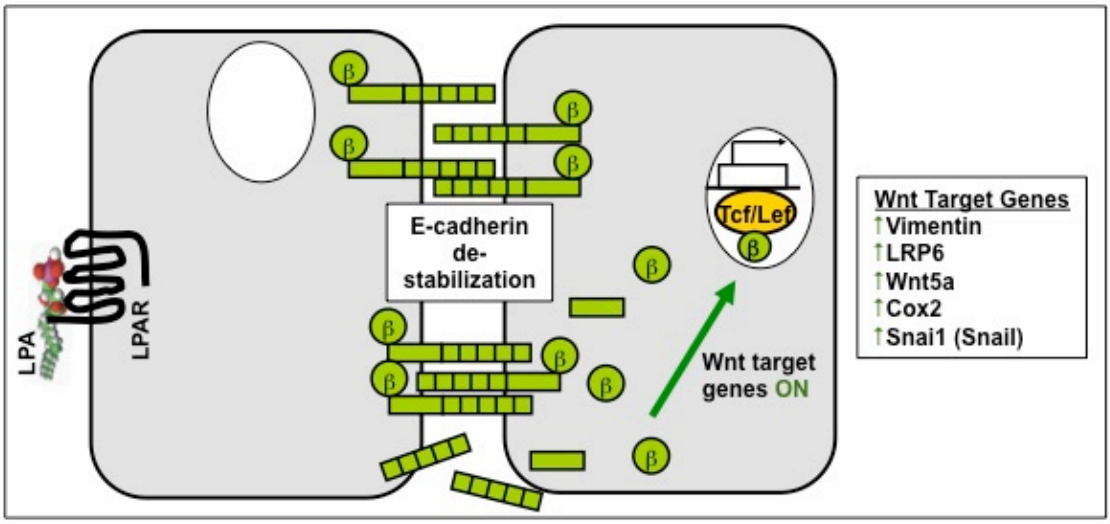


Figure 3.9: Chapter 3 Summary. LPA-mediated dissolution of adherens junctions leads to increased nuclear β-catenin accumulation, and subsequent activation of pro-metastatic Wnt/β-catenin target gene expression.

4. INTEGRIN-MEDIATED MATRIX ENGAGEMENT ACTIVATES β-CATENIN SIGNALING

I. RATIONALE

During the ovarian carcinoma metastatic cascade, disseminating cells anchor to the mesothelium and submesothelial matrix of peritoneal organs [Hudson et al, 2008; Barbolina et al, 2009]. Although mechanisms regulating mesothelial receptivity have not been fully investigated, mechanical deformation of the mesothelium caused by accumulated ascites fluid may expose submesothelial matrix and/or modulate surface-expressed proteins on mesothelial cells themselves [Zareie et al, 2005]. It has also been shown that adhesion of epithelial ovarian carcinoma (EOC) cells to the mesothelial monolayer itself can induce mesothelial cell retraction [Niedbala et al, 1986]. Interestingly, recent data suggests that EOC cells generate integrin-dependent traction force to physically displace mesothelial cells, subsequently adhering to the

submesothelial matrix beneath [Iwanicki et al, 2011]. In addition to anchoring to the submesothelial matrix, a fibronectin-rich, adhesion-promoting stroma is deposited on the otherwise non-adhesive mesothelium that may facilitate EOC cell adhesion [Nagy et al, 1995; Kenny et al, 2009].

Free-floating ovarian tumor cells adhere to the mesothelium and collagen-rich submesothelial matrix in order to form metastatic lesions, and this adhesion is in part mediated by β1 integrin [Cannistra et al, 1995; Strobel and Cannistra, 1999; Lessan et al, 1999]. Upon engagement with these intact matrices (multivalent ligand), integrin heterodimers aggregate (or cluster) and trigger downstream signaling events that differ from signaling events downstream of fragmented matrix (monovalent ligand). Ligation of integrins by monovalent ligand mediates receptor re-localization to focal contacts, but little tyrosine phosphorylation-regulated signaling. Conversely, integrin engagement of multivalent ligands or interaction with a non-ligand aggregator leads to FAK phosphorylation and cytoskeletal rearrangement (**Fig. 1.2**, **Fig. 4.1A**) [Miyamoto et al, 1995]. Previous work in our laboratory has modeled sub-mesothelial anchoring of metastasizing epithelial ovarian carcinoma cells microsphere-immobilized β1 integrin antibodies to mimic matrix-induced integrin aggregation [Ghosh et al, 2005; Symowicz et al, 2007]. Based on those findings, this work utilizes microsphere-immobilized anti-β1 integrin antibodies that recapitulate multivalent ligand-induced signaling to accurately mimic tumor cell adhesion to the mesothelium and/or submesothelial matrix (**Fig 4.1B**).

Epithelial (E)-cadherin is a single-span transmembrane glycoprotein that mediates calcium-dependent cell-cell adhesion via interaction with the extracellular domains of cadherins on the surface of neighboring cells (Gumbiner et al, 2000; Foty et al, 2005]. Although most normal epithelia express high levels of E-cadherin, it is absent in the mesenchymally-derived normal ovarian surface epithelium (OSE), which instead expresses neural (N)-cadherin [Hudson et al, 2008]. In most carcinomas, E-cadherin expression is downregulated or lost, facilitating cellular dispersal, invasion and metastasis [Hanahan and Weinberg, 2000]. However, a unique feature of EOC is a gain of E-cadherin expression in primary differentiated carcinomas, with all histotypes displaying strong immunoreactivity [Hudson et al, 2008]. There is less clarity regarding relative E-cadherin levels during ovarian tumor progression and metastasis. While complete loss of E-cadherin is uncommon, reduced staining is often detected in late stage tumors and in ascites-derived tumor cells [Davies et al, 1998; Ho et al, 2006], and negative E-cadherin is predictive of poor overall survival [Voutilainen et al, 2006; Darai et al, 1997].

β-catenin is found predominantly in association with the E-cadherin cytoplasmic domain at cell-cell junctions [Daugherty et al, 2007; Liu et al, 2002]. In the absence of cell-cell contact and Wnt signaling, cytosolic β-catenin forms a complex with adenomatous polyposis coli (APC), axin/conductin, casein kinases (CK) 1α and 1ε, and glycogen synthase kinase-3β (GSK-3β). CK1 and GSK-3β

phosphorylate β-catenin and target the protein for ubiquitination and proteasomal degradation [Liu et al, 2002]. When Wnt signaling is active, Dishevelled prevents complex formation and phosphorylation by GSK-3β, enabling β-catenin to translocate to the nucleus, bind Tcf/Lef-1 family transcription factors, and transcriptionally regulate Wnt/β-catenin target genes [Nelson and Nusse, 2004]. The transcriptional regulatory activity of β-catenin is also controlled by factors other than Wnt signaling. For example, ectopic E-cadherin expression can sequester β-catenin, thereby depleting the pool that binds Tcf [Gottardi et al, 2001]. Phosphorylation of GSK-3β by protein kinases A, B, and C, Akt/PI3K, and MAPK, inhibits its ability to phosphorylate and target β-catenin for degradation [Fang et al, 2000; Fang et al, 2002; Zhou et al, 2004].

Many Wnt/β-catenin target genes regulate tumor progression [Nelson and Nusse, 2004], however gene mutations in the Wnt signaling pathway are uncommon in ovarian cancer with the exception of some β-catenin mutations in endometrioid histotype EOC. Nevertheless, emerging data implicate dysregulated Wnt signaling in EOC progression in the absence of activating mutations in either APC, AXIN or β-catenin [Gamallo et al, 1999; Wright et al, 1999; Wu et al, 2001; Gatcliffe et al, 2008]. Several studies have also demonstrated a link between integrin signaling and β-catenin-dependent Wnt pathway activation in physiologic and pathologic cell signaling processes [Crampton et al, 2009; Maher et al, 2009; Kim et al, 2009; Chandramouly et al, 2007; Koenig et al, 2006; Barbolina et al, 2009]. Furthermore, both E-cadherin ectodomain shedding [Maretsky et al,

2005] and decreased net E-cadherin expression [Koenig et al, 2006] can promote β-catenin-mediated transcription, suggesting that β-catenin is released from E-cadherin following disruption of the junctional complex. These studies describe a novel mechanism by which cell-matrix engagement may regulate the functional integrity of cell-cell contacts, leading to increased β-catenin nuclear signaling and enhanced invasive activity.

II. RESULTS

Integrin Aggregation Alters β-catenin Dynamics.

β-catenin is commonly found in association with the E-cadherin cytoplasmic domain at cell-cell junctions [Gottardi and Gumbiner, 2004]. Previous data has shown intersection between junctional (E-cadherin-associated) and nuclear β-catenin pools [Kam and Quaranta, 2009]. Cytosolic β-catenin can be targeted for degradation or translocated to the nucleus. In the absence of Wnt signaling activation, GSK-3β phosphorylates β-catenin when it is complexed with APC and Axin and targets it for ubiquitination and degradation,preventing translocation to the nucleus [Dihlmann and von Knebel Doeberitz, 2005]. Our laboratory has shown that while total GSK-3β expression levels were unaffected, GSK-3β inhibition (Ser9 phosphorylation) was enhanced following integrin clustering [Burkhalter et al, 2011].

To examine whether integrin clustering may expand the pool of transcriptionally active β-catenin, nuclear extracts were isolated by differential centrifugation and analyzed for presence of β-catenin. Results show that integrin clustering

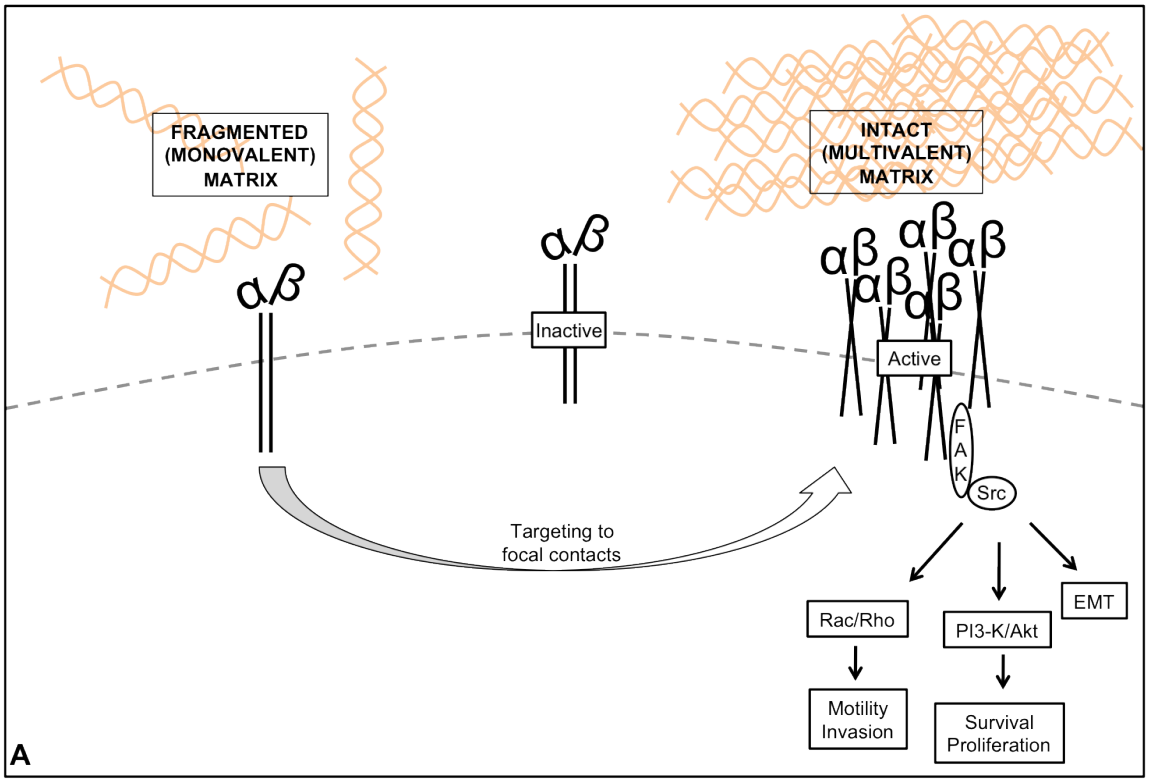
enhances nuclear accumulation of β-catenin (**Fig. 4.2A**). This was confirmed using cytospin/immunofluorescence analysis in which cell are lysed in detergent-free hypotonic buffer, intact nuclei are collected and centrigfuged onto poly-L-lysine-coated coverslips using a Cytopro cytocentrifuge. Nuclei were then stained β-catenin or active-β-catenin by immunofluorescence as described in Experimental Methods. These data demonstrated an increase in nuclear β-catenin fluorescence (**Fig. 4.2B**).

Integrin Aggregation Activates Transcription of Wnt/β-catenin Target Genes.

As the data above indicated that integrin clustering altered β-catenin dynamics, resulting in enhanced nuclear β-catenin levels, cells were evaluated for changes in β-catenin-regulated gene expression. This was confirmed by qPCR profiling of Wnt pathway genes that are targets of β-catenin; Seventy-five genes encoding Wnt pathway members were upregulated in response to integrin clustering as shown as a scatter plot (**Fig. 4.3A**) and as a heat map where red indicates upregulation, black indicates no change and green indicates downregulation (**Fig. 4.3B**). Each gene tested and its fold-change expression is listed in **Table 4.1**. Validation of select genes from the PCR profiling data demonstrates significantly enhanced expression of *PTGS2* (Cox-2, p-value= 0.0108), *PLAUR* (uPAR, p-value= 0.0144), *VIM* (vimentin, p-value= 0.0097), *LRP6* (p-value=

0.0017), *WNT5A* (p-value= 0.0001) and *MMP9* (p-value= 0.0287) and increased expression of snail (p-value= 0.06) (**Fig. 4B**).

4.1 MODELING INTEGRIN CLUSTERING – MONOVALENT VERSUS MULTIVALENT MODELS



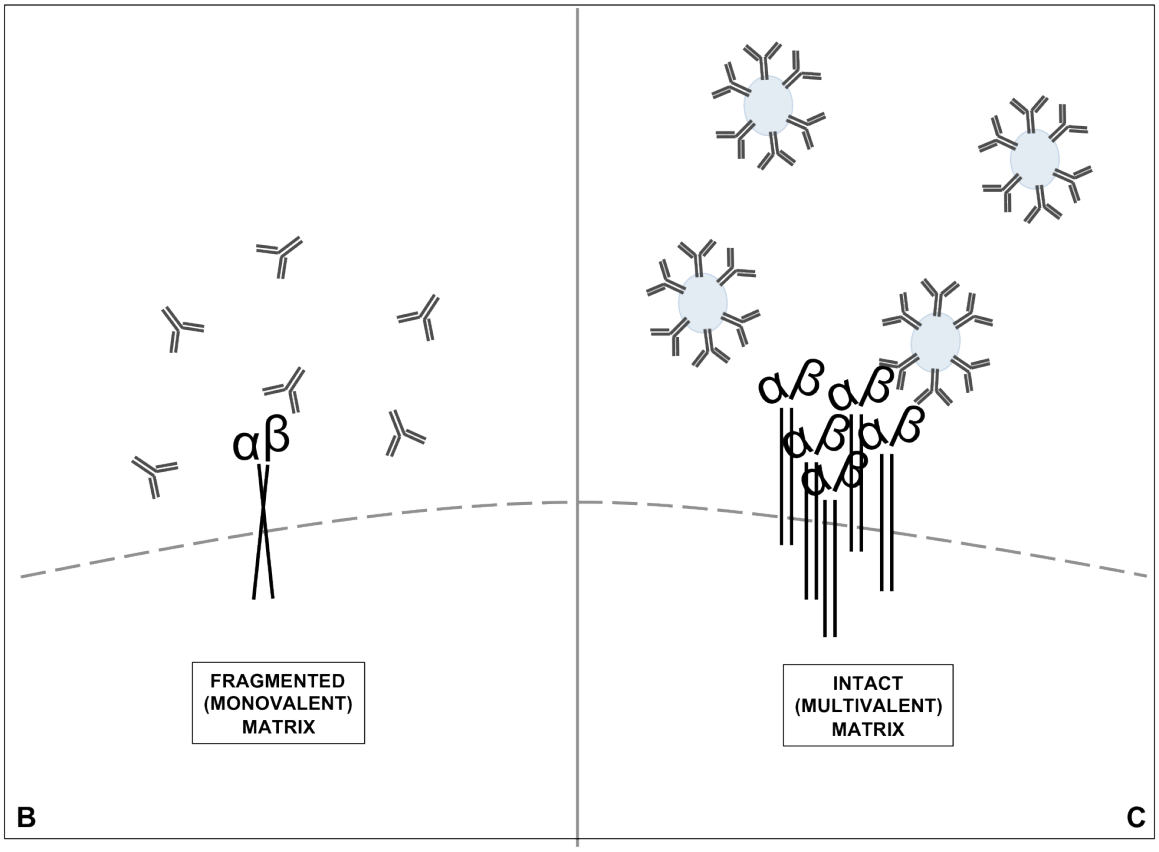
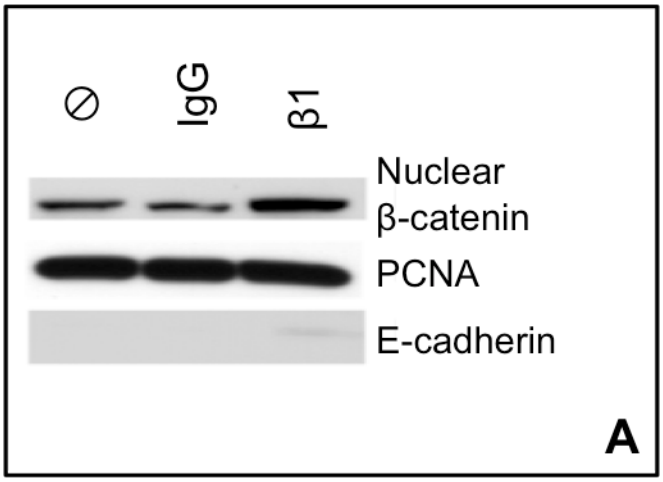


Figure 4.1: Modeling Integrin Clustering – Monovalent versus Multivalent Models. A) Multivalent substrate ligation (intact matrix) of integrins activates FAK-dependent signaling responses which differ from monovalent ligation, or fragmented matrix. Integrin aggregation-induced signaling mediates a number of cellular processes in ovarian carcinoma, including epithelial-to-mesenchymal transition, cell motility and invasion, proliferation and cell survival. Previous studies have utilized soluble anti-β1 integrin antibody solutions, which mimics integrin interaction with fragmented substrate (B). In order to model cell adherence to intact substrate, anti-β1 antibodies are adsorbed to 3-micron polystyrene microspheres (described in Experimental Methods, C).

4.2 ENGAGEMENT OF β1 INTEGRINS INCREASES NUCLEAR β-CATENIN



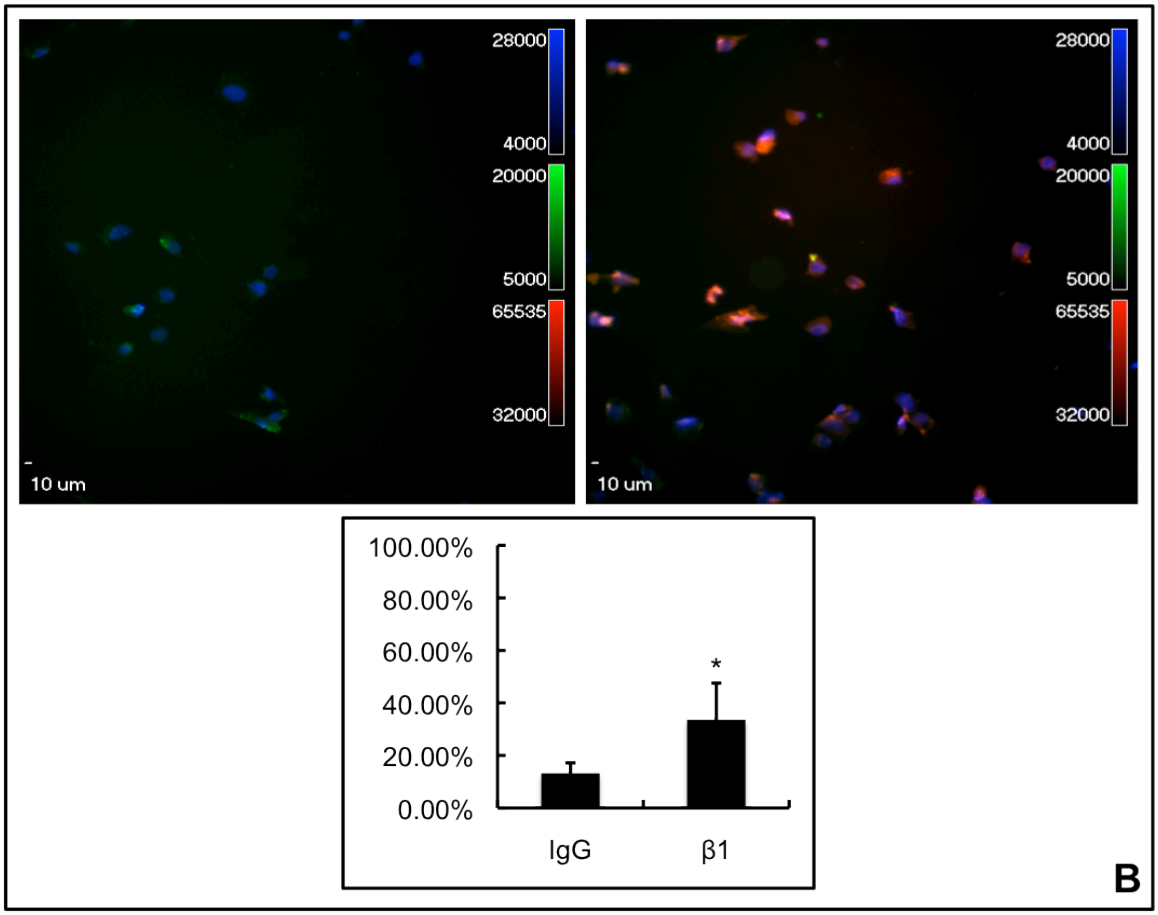
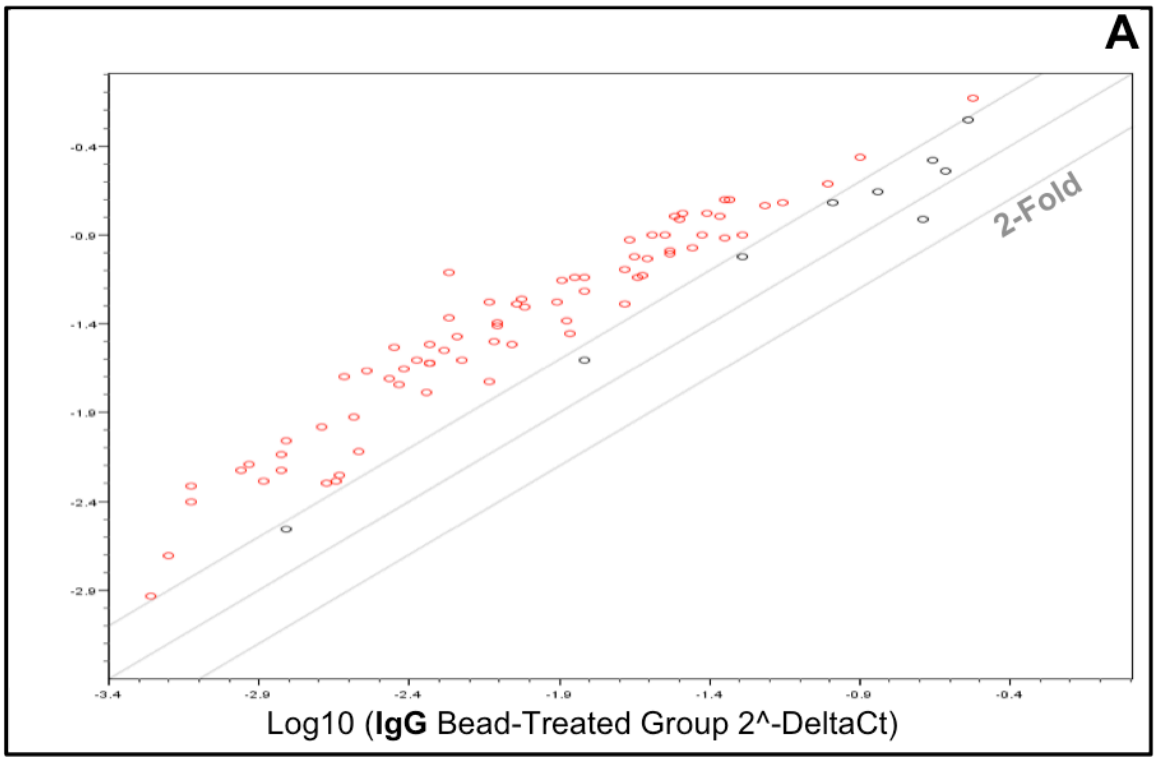


Figure 4.2: Engagement of β1 integrins increases nuclear β-catenin. (A) Cells were treated with microsphere-immobilized anti-integrin-β1 (TS2) or control IgG (as indicated) for 90 minutes. Nuclear extracts were isolated as described in Experimental Procedures and electrophoresed on a SDS-PAGE gel, transferred to a PVDF membrane and immunoblotted with anti-β-catenin (1:1000) followed by peroxidase-conjugated secondary antibody (1:5000) and enhanced chemiluminescence detection. Proliferating cell nuclear antigen (PCNA) was used as a control nuclear protein while E-cadherin was used to show absence of non-nuclear proteins. (B) Cells were treated with microsphere-immobilized anti-integrin β1 or control IgG for 90 minutes, then immediately lysed by hypotonic shock. Intact nuclei were collected by cytospin and evaluated for β-catenin using fluorescently tagged anti-β-catenin antibodies (1:200 dilution). Nuclei are counterstained with DAPI. Red – anti-active β-catenin-AlexaFluor649; green – anti-β-catenin-AlexaFluor488; blue – DAPI. Lower panel shows quantitation of positive nuclei from three different experiments.
*p=.001

4.3 ENGAGEMENT OF β1 INTEGRINS ALTERS GENE EXPRESSION OF WNT PATHWAY MEMBERS



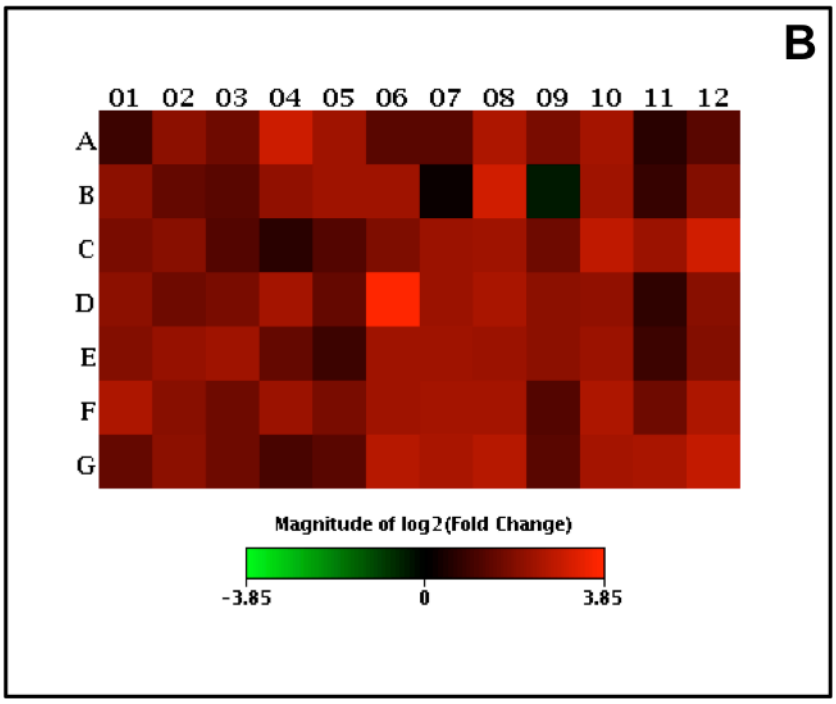


Figure 4.3: Engagement of β1 Integrins Alters Gene Expression of Wnt Pathway Members. Cells were treated with microsphere-immobilized anti-integrin-β1 or control IgG. RNA was isolated using Trizol reagent, and a cDNA library was prepared using RT^2 First Strand Kit (SA Biosciences). The Wnt RT-qPCR array (PAHS-043, SA Biosciences) was performed according to manufacturer's specifications and utilized internal controls. Red designated genes upregulated in cells treated with anti-integrin-β1 microspheres relative to controls. Data is represented as a scatterplot (A) and as a heat map (B).

4.1 ENGAGEMENT OF β1 INTEGRINS ALTERS EXPRESSION OF WNT SIGNALING GENES

	01	02	03	04	05	06	07	08	09	10	11	12
A	AES 1.87	APC 4.29	AXIN1 3.14	BCL9 8	BTRC 5.1	FZD5 2.46	CCND1 2.46	CCND2 5.86	CCND3 3.36	CSNK1A1 5.46	CSNK1D 1.52	CSNK1G1 2.55
B	CSNK2A1 4.29	CTBP1 2.73	CTBP2 2.46	CTNNB1 4.44	CTNNBIP 1 5.1	CXXC4 5.1	DAAM1 1.19	DIXDC1 8.28	DKK1 0.76	DVL1 5.28	DVL2 1.8	EP300 3.86
C	FBXW11 3.48	FBXW2 4	FGF4 2.38	FOSL1 1.52	FOXN1 2.3	FRAT1 3.61	FRZB 4.92	FSHB 5.28	FZD1 3.03	FZD2 7.21	FZD3 4.76	FZD4 8.28
D	FZD6 4.29	FZD7 3.03	FZD8 3.48	GSK3A 5.46	GSK3B 2.73	JUN 14.42	KREMEN1 4.92	LEF1 5.66	LRP5 4.14	LRP6 4.44	MYC 1.62	NKD1 4
E	NLK 3.73	PITX2 4.59	PORCN 5.1	PPP2CA 2.73	PPP2R1A 1.93	PYGO1 5.1	RHOU 5.1	SENP2 4.92	SFRP1 4.29	SFRP4 4.92	FBXW4 1.87	SLC9A 3.73
F	SOX17 5.86	T 4	TCF7 3.14	TCF7L1 4.92	TLE1 3.36	TLE2 5.1	WIF1 5.46	WISP1 5.46	WNT1 2.38	WNT10A 5.86	WNT11 3.14	WNT16 5.86
G	WNT2 2.83	WNT2B 4.14	WNT3 3.14	WNT3A 2.14	WNT4 2.55	WNT5A 6.5	WNT5B 5.66	WNT6 6.5	WNT7A 2.46	WNT7B 5.46	WNT8A 5.66	WNT9A 7.46

Table 4.1 Engagement of β1 Integrins Alters Expression of Wnt Signaling Genes. Fold change of each gene compared with GAPDH. Red designated genes upregulated at least two-fold in cells treated with anti-β1 microbeads relative to controls.

4.4 INTEGRIN CLUSTERING UPREGULATES TRANSCRIPTION OF β-CATENIN TARGET GENES

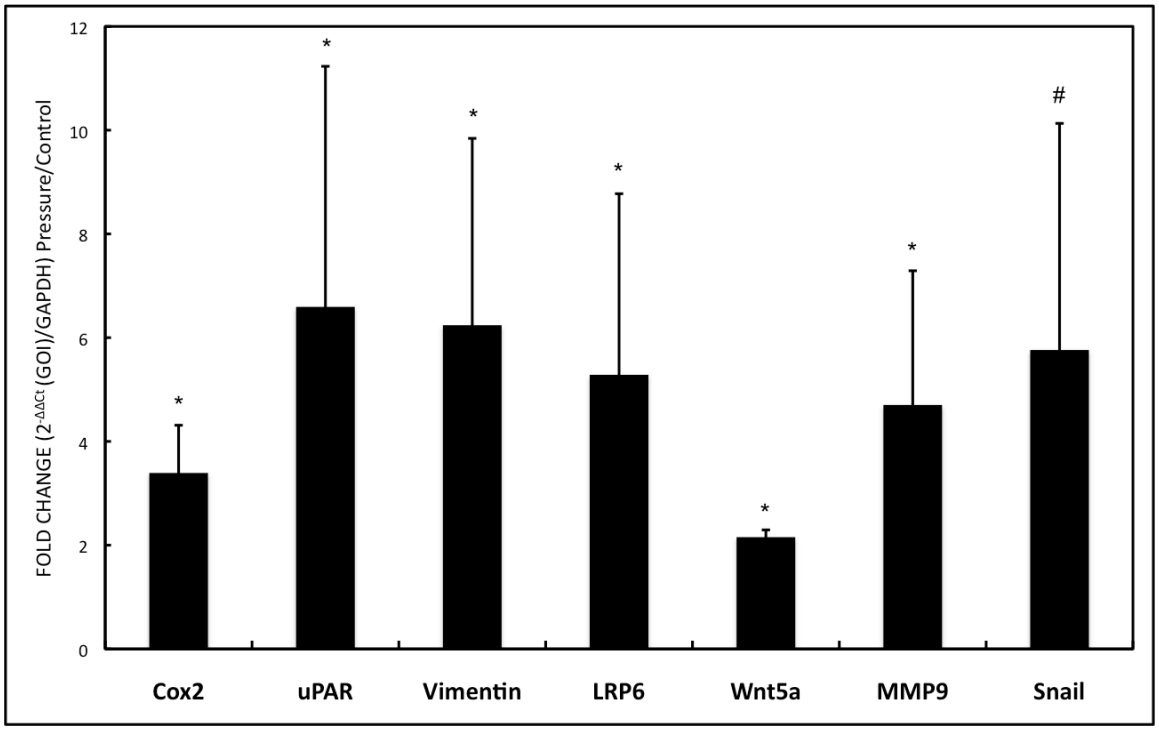


Figure 4.4: Integrin Clustering Upregulates Transcription of β-catenin Target Genes. Cells were treated with microbead-immobilized anti-integrin β1 or control IgG prior to isolation of RNA and evaluation of Wnt target gene expression by real time RT-PCR as described in Experimental Methods. Results represent the mean of a minimum of four independent experiments.
*p<.05; #p=0.1

III. DISCUSSION

Modulation of cell-cell and cell-matrix adhesion are key events in ovarian cancer metastasis, as intra-peritoneal adhesion of malignant cells and multi-cellular aggregates combined with localized integrin-mediated invasion of the collagen-rich sub-mesothelial matrix are necessary to anchor secondary lesions [Hudson et al, 2008; Brabletz et al, 2005]. Intra-peritoneal ovarian cancer metastasis is mediated by adhesion via integrins α2β1 and α3β1 to peritoneal mesothelial cells displaying surface expression of collagen and the exposed interstitial (types I and III) collagen-rich submesothelial matrix and antibodies directed against these integrins block collagen binding [Gottardi et al, 2004; Srivastava and Pandey, 1998; van Noort et al, 2002; Tago et al, 2000; Barbolina et al, 2009; Skubitz et al, 2002; Casey and Skubitz, 2000]. Integrin engagement by a multivalent matrix ligand results in receptor aggregation, functionally coupling the extracellular environment to specific signal transduction pathways that modulate distinct cellular responses including gene transcription, cell migration, and survival [Lessan et al, 1999]. Integrins thereby signal cellular responses by regulating the formation of signal transduction complexes on a cytoskeletal framework and this integration of signaling and cytoskeletal events is dictated by the physical nature of the integrin-ligand interaction [Lessan et al, 1999].

Recent studies have shown that extracellular matrix (ECM) can modulate Wnt signaling. Specifically, the ECM component biglycan may form a heterotrimer with Wnt ligands and the co-receptor LRP6, activating β-catenin-dependent transcription [Berendsen et al, 2011]. The current results show that engagement of collagen-binding integrins enables β-catenin activation, nuclear translocation, and transcriptional regulation. Increased β-catenin nuclear signaling enhances transcription of many genes that contribute to tumor progression [Dihlmann and von Knebel Doeberitz, 2005; Nelson and Nusse, 2004] including genes that modulate invasion and metastasis [Ellerbroek et al, 1999]. Detection of Wnt target gene expression provides additional evidence in support of Wnt pathway activation. Enhanced expression of *COX2*, *PLAUR* (uPAR), *VIM* (vimentin), *LRP6*, *WNT5A*, *MMP9* and *SNAI1* (snail 1) was demonstrated in the current study. The membrane-anchored receptor uPAR binds urinary-type plasminogen activator (uPA) and participates in cell surface-associated plasminogen activation. The uPA-uPAR-plasmin system has been linked to invasion and metastasis in multiple tumor types and elevated uPAR has been shown to contribute to proliferation, adhesion and invasion [Ahmed et al, 2005]. Expression of uPAR is elevated in human ovarian tumors and correlates with tumor stage, grade and unfavorable prognosis with respect to disease progression free survival [Young et al, 1994]. As previously described (Chapter 3), Wnt5a, Cox-2, MMP-9, LRP6 and Snail 1 also play important roles in ovarian cancer progression.

The current data describe a mechanism for integrin-dependent activation of Wnt/β-catenin signaling that may play a role in metastatic progression. Integrin clustering alters β-catenin dynamics, inducing nuclear translocation of β-catenin and transcriptional activation of Wnt/β-catenin target genes. While some of these genes may also be activated by other pathways, the current data support the hypothesis that matrix-induced integrin clustering may provide a novel mechanism for the dysregulation of Wnt signaling observed in ovarian tumors lacking mutations in the Wnt signaling pathway [Wu et al, 2001; Gamallo et al, 1999; Wright et al, 1999; Gatcliffe et al, 2008].

4.5 CHAPTER 4 SUMMARY

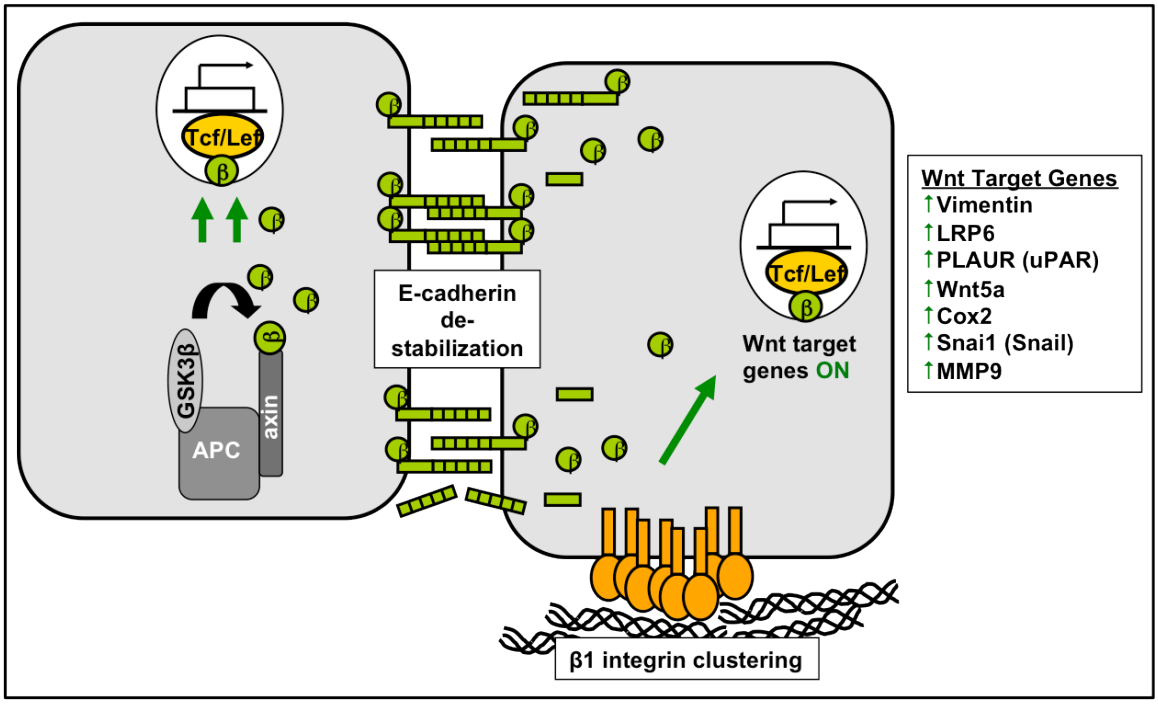


Figure 4.5: Chapter 4 Summary. Integrin engagement-induced adherens junction destabilization leads to nuclear localization of β-catenin, and subsequent activation of Wnt signaling target genes.

5. LYSOPHOSPHATIDIC ACID ACTIVATES β1 INTEGRIN IN OVARIAN CARCINOMA

I. RATIONALE

Many studies have suggested convergent signaling between growth factors and integrin-mediated adhesion processes [reviewed in Mitra et al, 2005, Alam et al, 2007, Harburger and Calderwood, 2009]. Several G-protein coupled agonists, including lysophosphatidic acid (LPA), have been shown to phosphorylate the integrin effector p125FAK [Rozengurt, 1995]. Additionally, LPA-induced migration in fibroblasts is dependent on β1 integrin expression [Sakai et al, 1999]. One study supports a theory of LPA-integrin crosstalk by demonstrating that LPA-$LPAR_2$ interaction activates TGF-β signaling in an integrin-mediated manner [Xu et al, 2009].

In ovarian cancer, data has suggested crosstalk between the LPA and integrin signaling pathways in epithelial ovarian carcinoma (EOC). EOC cells cultured on

laminin demonstrate increased LPA production; treatment with an inhibitory anti-β1 integrin antibody abrogates LPA production and cell adhesion to laminin. Further, addition of exogenous LPA following anti β1 antibody pre-treatment restores EOC cell adhesion to laminin [Sengupta et al, 2003]. Interestingly, LPA treatment also leads to increased adhesion to collagen I in ovarian cancer cell lines, as well as enhanced β1 integrin protein expression [Fishman et al, 2001].

Ovarian tumor metastatic success is achieved in part due to the ability to survive anoikis: programmed cell death due to lack of or inappropriate cell adhesion [Frankel et al, 2001]. Specificity for extracellular matrix component(s) is conferred to cells by integrin heterodimer expression, and cells adhered to inappropriate matrices are equally susceptible to anoikis as detached cells [Frisch and Ruoslahti, 1997]. β1 integrin has been shown to rescue cells from anoikis through tyrosine phosphorylation of Shc, and subsequent MAP kinase signaling in T lymphocyte and osteosarcoma cell lines [Wary et al, 1996]. It has also been suggested that LPA confers anoikis-resistance to metastasizing ovarian tumor cells through hyperphosphorylation of p125FAK [Erickson et al, 2001], suggesting potential crosstalk between LPA and integrins. These data taken together support the concept that both integrin signaling and LPA signaling promote cell survival in part through regulating programmed cell death. Whether integrin and LPA signaling mechanisms are cooperative or involve crosstalk between the two pathways is unclear.

Results reported in Chapters 3 and 4 include integrin clustering- and LPA-induced translocation of cytoplasmic β-catenin to the nucleus of ovarian carcinoma cells, and subsequent activation of beta-catenin-dependent gene transcription. These similarities suggest a potential for cross or combinatory signaling between β1 integrin and LPA, and the subsequent experiments were designed to investigate potential for convergence of the integrin and LPA signaling pathways that potentiate ovarian tumor progression.

II. RESULTS

Lysophosphatidic Acid Activates Clustering-Dependent β1 Integrin Signaling.

Lysophosphatidic acid (LPA) – LPA receptor interaction has been previously shown to transactivate a number of surface-expressed signaling receptors such as EGFR and VEGF [Choi et al, 2010]. In order to assess the potential for cross-talk between LPA signaling and integrin activation, OVCA433 cells were treated with either 30 μM or 70 μM LPA for 1 hour at 37°C, and then evaluated for β1 integrin activation by flow cytometry analysis. Adherent monolayer cells were treated with LPA, followed by immuno-labeling with an antibody that specifically recognizes the active conformation of β1 integrin (anti-β1 integrin, clone HUTS21, BD Transduction Laboratory). Both 30 and 70 μM LPA treatments induced β1 integrin activation, and this activation was partially decreased by pre-treatment with an LPA receptor inhibitor, Ki16425 (**Fig. 5.1A, B**). Disseminating ovarian tumor cells are exposed to LPA in ascites as anchorage-independent multicellular aggregates (MCAs). In order to evaluate β1 integrin clustering in anchorage-independent cells in response to LPA, OVCA433 cell suspensions were treated with 20 μM or 40 μM LPA for 1 hour at room temperature. Surface-

expressed β1 integrin was crosslinked using a non-activating anti-β1 integrin (clone MAB1959) antibody (Millipore) at 4°C, followed by incubation with anti-IgG (Sigma-Aldrich) at 37°C. The cell suspension was diluted 1:10 in serum-free medium, and then cyto-centrifuged onto 22 mm^2 glass coverslips, and immunostained for β1 integrin (anti-β1 integrin, clone MAB2250; secondary antibody: Alexa fluor 488-conjugated mouse-anti IgG) as illustrated in **Fig. 5.2**. Data was quantitatively evaluated by counting the number cells exhibiting punctate green spots [Gilcrease et al, 2004] compared with the total number of cells in the field of view (40X magnification). β1 integrin clustering was potentiated by LPA treatment in a dose-dependent manner, with a 1.5-fold increase of the number of β1 integrin clusters (green) following 20 μM treatment (**Fig. 5.3B,D**) and a 2-fold increase of clusters following 40 μM treatment (**Fig 5.3C,D**) compared with control (**Fig. 5.3A**).

Clustering-dependent integrin signaling activates differential signaling compared with substrate-ligation of individual integrin dimers, including FAK and Src phosphorylation [Miyamoto et al, 1995], as illustrated in **Fig 1.2**. Clustering-induced signaling activation is initiated by autophosphorylation of FAK at tyrosine 397, which is a binding site for the Src family of kinases. Further, phosphorylation of FAK^{Tyr397} and recruitment of Src kinases leads to phosphorylation of tyrosine 576, which lies in the FAK activating domain. [Schaller et al, 1994; Cobb et al, 1994; Chen et al, 1996; Zhang et al, 1999]. Src catalytic activity is regulated by tyrosine phosphorylation at two different sites

with contrasting consequences; activation requires autophosphorylation at tyrosine 418, whereas phosphorylation of tyrosine 527 decreases enzyme activity [Hunter et al, 1987]. Phosphorylation status of FAK and Src were evaluated following LPA treatment, to determine whether LPA-induced clustering also activates integrin signaling. LPA treatment led to phosphorylation of FAK at tyrosine residues 576 and 397 in a LPA receptor dependent manner, suggesting LPA activates FAK downstream of β1 integrin clustering (**Fig. 5.4A,B**). Corresponding to LPA-induced FAK activation, Src is phosphorylated at tyrosine 418 but not at tyrosine 527, indicating an increase of Src kinase activity (**Fig. 5.5A,B**).

Lysophosphatidic Acid Protects Disseminating Ovarian Tumor Cells From Anchorage-Dependent Apoptosis (Anoikis).

The studies above support the hypothesis that LPA regulation of β1 integrin may play a role in protect floating multicellular aggregates (MCAs) from anoikic cell death via modulation of integrin clustering and activation. The anoikis signaling pathway culminates with the activation of the caspase cascade [Valentijn et al, 2004].Anoikis can be initiated by either intrinsic (outer mitochondrial membrane permeabilization) or extrinsic (death signal, e.g. Fas, TNF-R) signaling pathways. Intrinsic anoikis is in part characterized by activation of caspase-8, while extrinsic anoikis is characterized by caspase-9 [Gilmore, 2005; Simpson et al, 2008]. Evaluation of caspase activation allows for determination relative rates of anoikic

cell death in response to LPA treatment. OVCA429 and OVCA433 cells were cultured as multicellular aggregates using the hanging drop method as described [Kelm et al, 2002; Experimental Methods], serum-starved overnight and transferred to agarose-coated dishes (to prevent adhesion), then treated with 1% BSA in PBS (control) or 40 μM LPA. In OVCA433, both caspase-8 (p-value=) and caspase-9 activity was decreased following treatment with LPA compared with control (**Fig. 5.6**). Activity of caspase-8 and caspase-9 trended towards decreasing in response to LPA in OVCA429, as well (**Fig. 5.7**).

5.1 LPA ACTIVATES β1 INTEGRINS

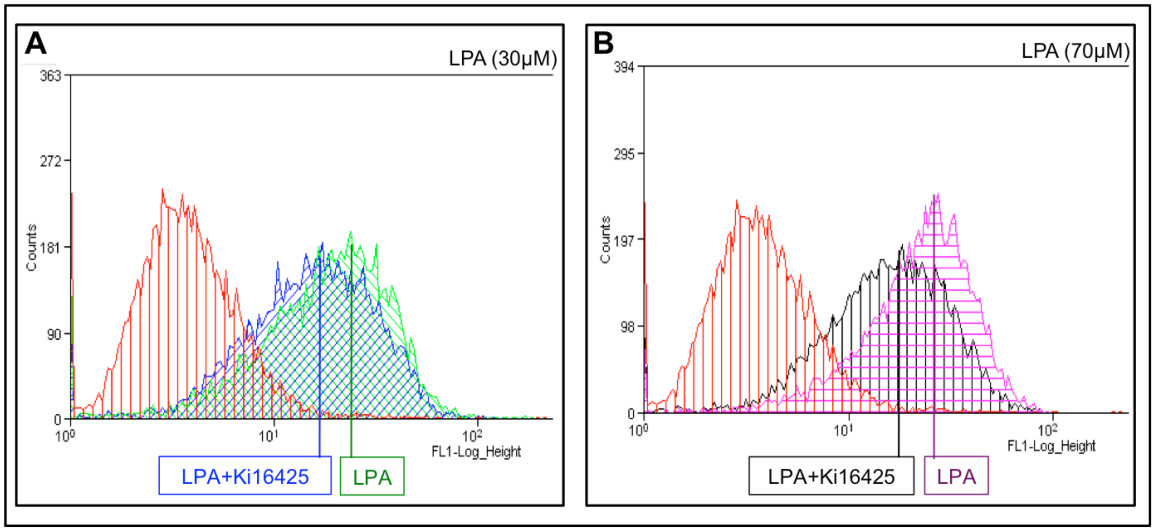


Figure 5.1: LPA Activates β1 Integrins. OVCA433 cells were serum-starved, pre-treated with 40 μM LPA receptor inhibitor, Ki16425, where indicated, then treated with indicated concentrations of LPA for 1 hour. Following incubation with anti-active-β1 integrin (1:100, 1 hour), and mouse anti-IgG-AlexaFluor 488 (1:500, 30 minutes), cells were analyzed for expression of active β1 integrin by flow cytometry. β1 integrin is activated in response to 30 μM (A, green) and 70 μM (B, purple) LPA. Inhibition of the LPA-LPA receptor interaction partially decreases β1 integrin activation (A, blue; B, black). Experiments were repeated three times for data validation.

5.2 IMMUNOFLUORESCENT EVALUATION OF β1 INTEGRIN CLUSTERING

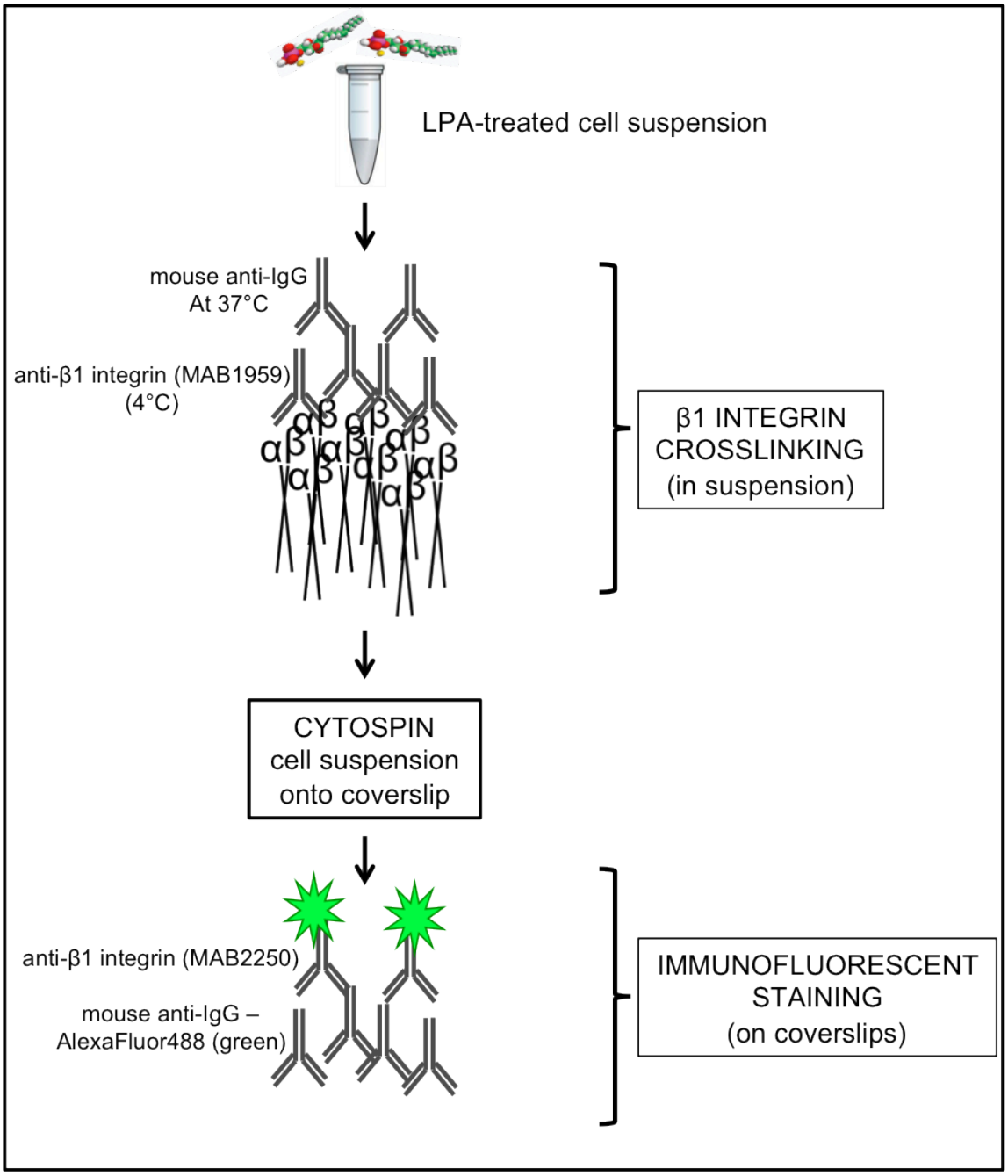


Figure 5.2: Immunofluorescent Evaluation of β1 Integrin Clustering. Suspended cells are treated with 40 μM LPA for 1 hour. Suspensions are then incubated with a function-blocking anti-β1 integrin antibody (clone MAB1959), on ice, following by a mouse anti-IgG antibody at 37°C to crosslink integrins. Cell suspensions are cytocentrifuged onto 22 mm^2 glass coverslips for immunofluorescent staining, and stained for β1 integrin (1°- anti-β1 integrin, clone MAB2250, 1:200; 2° mouse anti-IgG-Alexa Fluor 488, 1:500).

5.3 LPA INDUCES ANCHORAGE-INDEPENDENT β1 INTEGRIN CLUSTERING

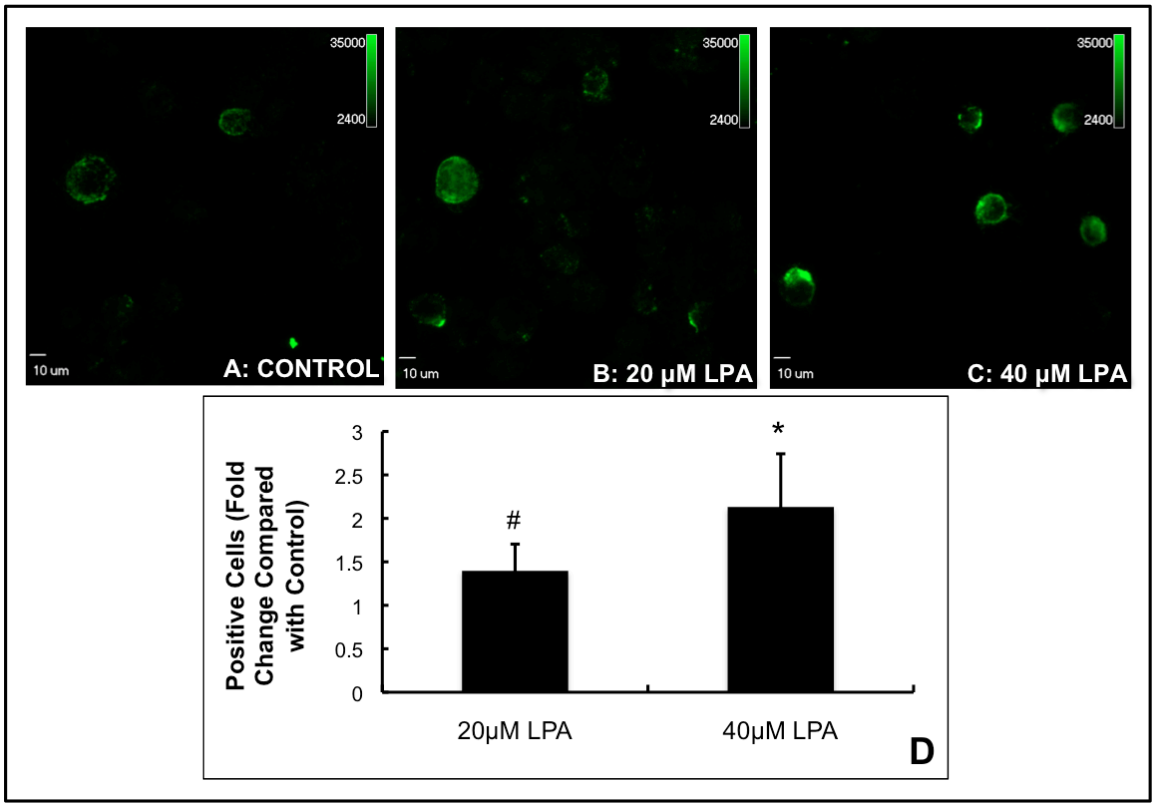


Figure 5.3: LPA Induces Anchorage-Independent β1 Integrin Clustering. OVCA433 cells were treated with 1% BSA in PBS (control, A), 20 μM LP,A or 40 μM LPA (C) as described (Figure 5.2, Experimental Methods), and analyzed using fluorescent microscopy. D) Positive cells were enumerated by counting the number cells exhibitng punctate spots (green); quantitative data is expressed as a mean of three experiments.
$^{\#}p<0.1$, $^{*}p<0.01$

5.4 LPA TREATMENT LEADS TO PHOSPHORYLATION OF FAK

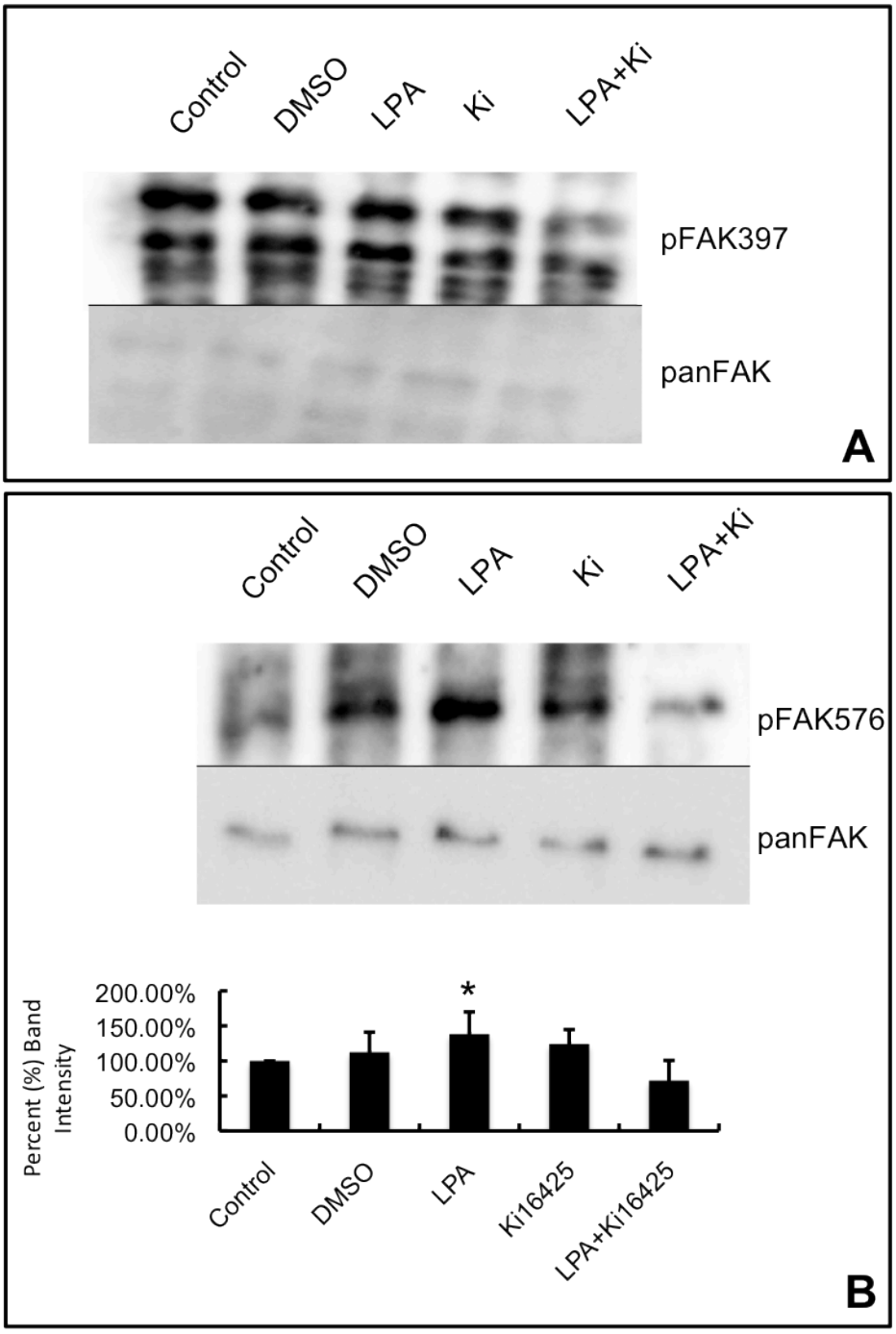


Figure 5.4: LPA Treatment Leads to Phosphorylation of FAK. OVCA433 cells were treated as indicated (40 μM LPA, 40 μM Ki16425) for 2 hours, and cell lysates were collected in mRIPA buffer. Samples were electrophoresed on a SDS-PAGE gel, transferred to a PVDF membrane and immunoblotted for pFAK576 (1:1000). PVDF membranes were stripped, then re-probed for panFAK (1:1000) as a control. LPA treament increased $pFAK^{397}$ (A) and $pFAK^{576}$ (B) expression *in vitro*, while total FAK expression was unchanged (B).
*$p<0.05$

5.5 LPA TREATMENT MEDIATES SRC PHOSPHORYLATION

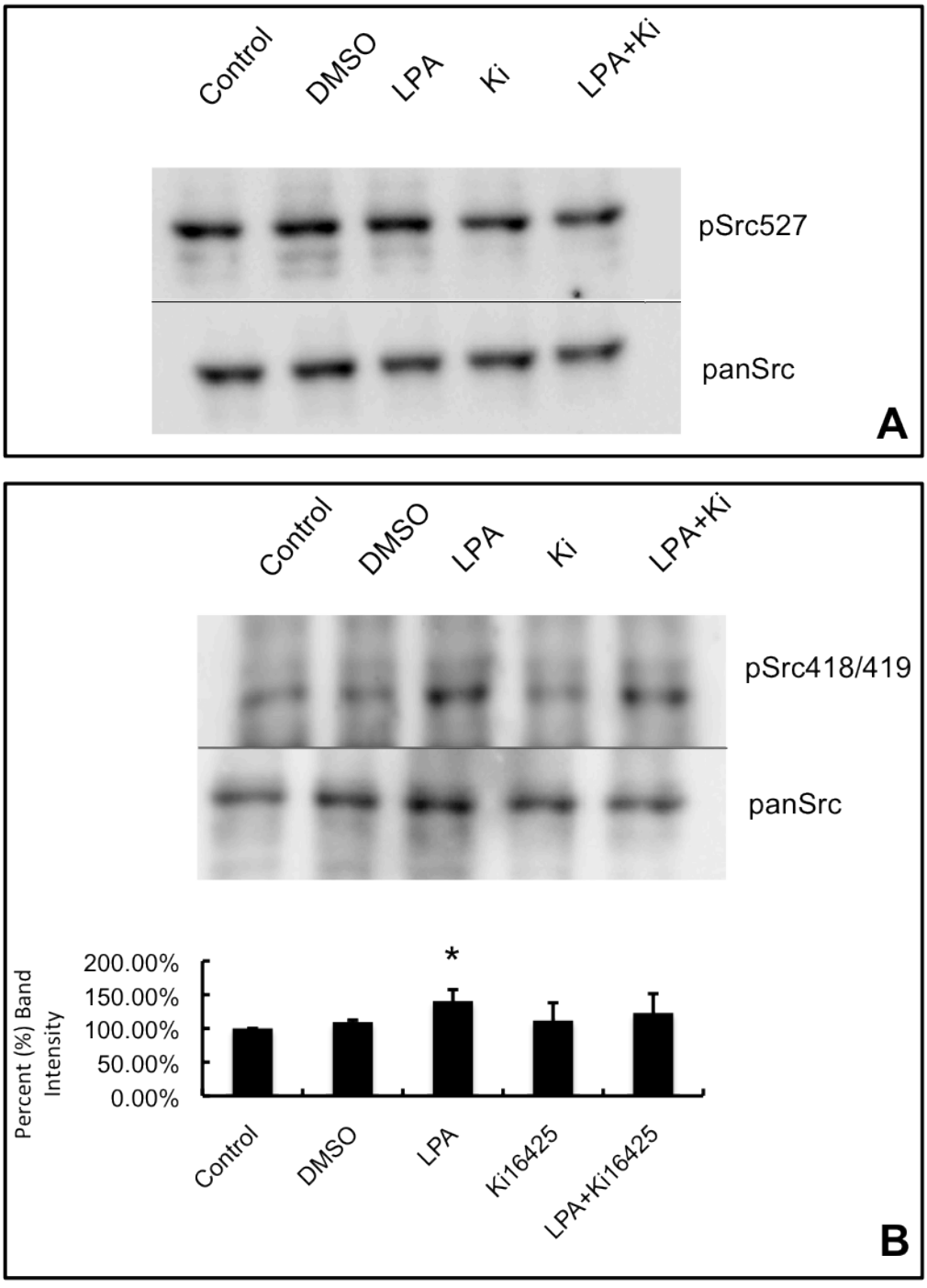


Figure 5.5: LPA Treatment Mediates Src Phosphorylation. OVCA433 cells were treated as indicated (40 μM LPA, 40 μM Ki16425) for 2 hours, and cell lysates were collected in mRIPA buffer. Samples were electrophoresed on a SDS-PAGE gel, transferred to a PVDF membrane and immunoblotted for pSrc418/419 (1:1000). PVDF membranes were stripped, then re-probed for panSrc (1:1000). The activating phosphoryatiion (Tyr418) was increased in response to LPA treatment (B), $pSrc^{527}$ (A) and pan Src (B) were unchanged.
*p<0.02

5.6 LPA DECREASES CASPASE ACTIVITY IN OVARIAN CARCINOMA CELL LINES

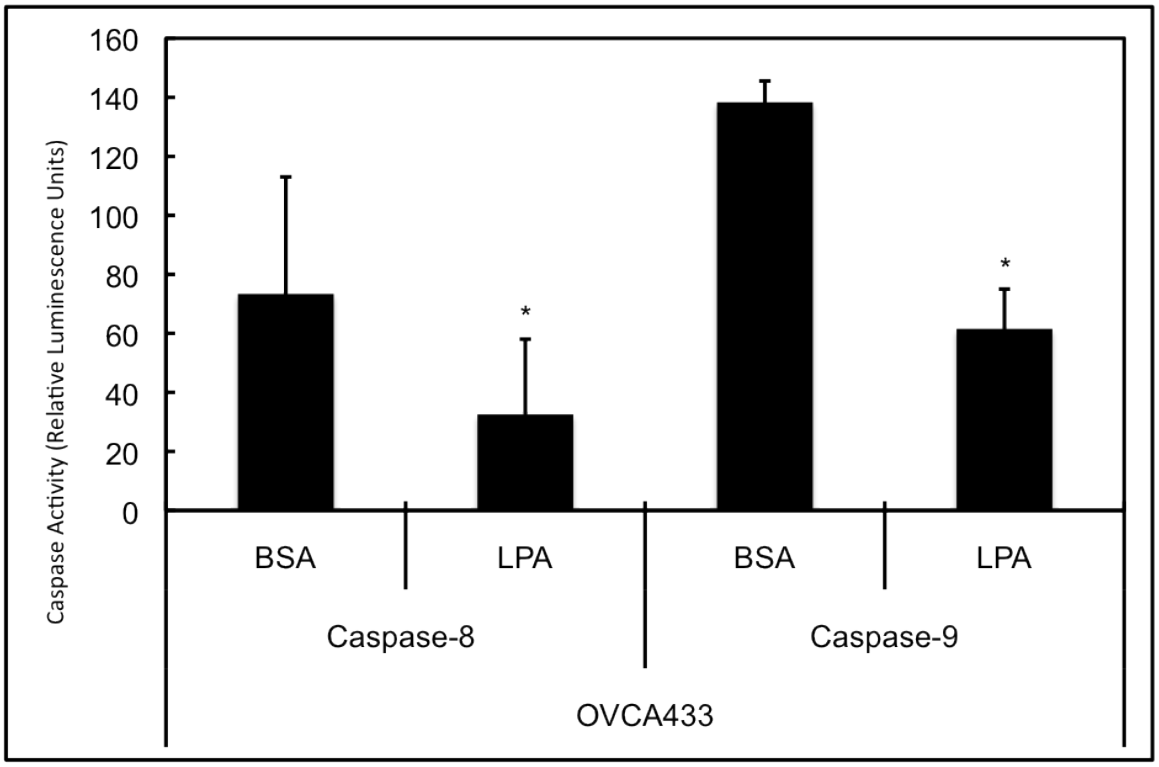


Figure 5.6: LPA Decreases Caspase Activity in OVCA433 Cells. Cells were treated with 40 μM LPA or 1% BSA in PBS, then analyzed for caspase-8 or -9 activity as described in Experimental Methods. LPA treatment decreased caspase-8 activity by 50% in OVCA433, and decreased caspase-9 by over 2-fold.
*$p<0.05$

5.7 LPA MAY MODULATE CASPASE ACTIVITY IN OVCA429 CELLS

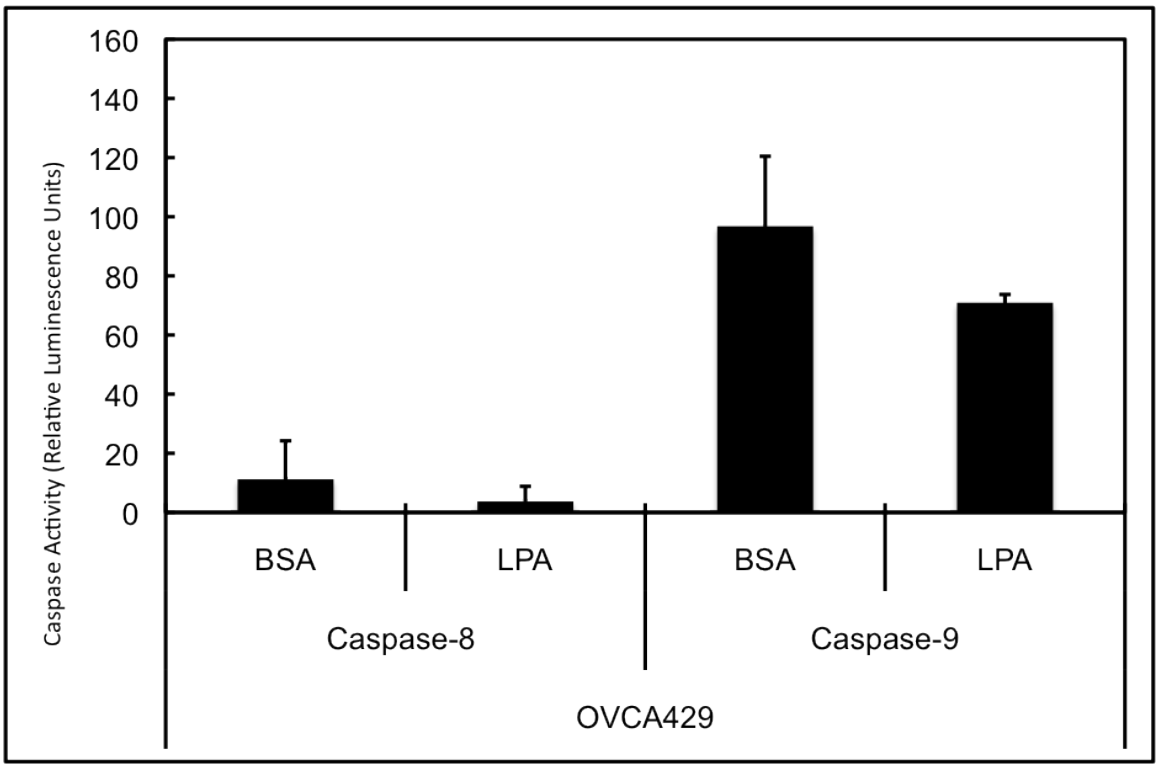


Figure 5.7: LPA May Moduate Caspase Activity in OVCA429 Cells. Cells were treated with 40 μM LPA or 1% BSA in PBS, then analyzed for caspase-8 or -9 activity as described in Experimental Methods. Data from LPA treatment of OVCA429 trended towards a decrease in caspase-8 and -9 activity.

III. DISCUSSION

Lysophosphatidic acid signaling regulates a diverse number of biological actions in nearly every system of the human body [Choi et al, 2010]. In addition to achieving influence by binding one of its five LPA receptors, LPA also transactivates other signaling pathways including growth factors, proteases and other factors that facilitate tumor progression and metastatic success [Fukushima et al, 2001; Choi et al, 2010; Wang and Fishman, 2009]. Although it has been suggested that growth factor-dependent cytoskeletal reorganization may potentiate integrin clustering in lipid rafts [Ross, 2004], cooperative signaling between LPA and integrins in ovarian cancer has not been elucidated. This chapter establishes pathway crosstalk between LPA signaling and integrin clustering-mediated signaling, demonstrates a role for LPA in protecting disseminating epithelial ovarian carcinoma (EOC) cell from anoikis (**Fig 5.8**).

Previous work has shown that LPA increases ovarian cancer cell line adhesion to Collagen type I via β1 integrin, and induces β1 integrin protein expression [Fishman et al, 2001]. Current data suggests this increased adhesion may be in part due to LPA-induced transition of β1 integrin to the active conformation. LPA

induces activation and clustering of integrins in OVCA433, an ovarian carcinoma cell line. Current models of integrin activation suggest that structurally β1 integrin activation is facilitated by talin- or kindlin-mediated disruption of alpha subunit and beta subunit cytoplasmic tails (**Fig. 4.1A**) [Hynes, 2002; Vinogradova et al, 2002; Askari e al, 2009; Nieves et al, 2010]. Activiy of talin itself is mediated by conformational change, which may be triggered by phosphatidylinositol (4,5)-biphosphate (PIP2). Interestingly, LPA has been shown to compete with PIP_2 in binding other proteins, such as gelsolin [Mintzer et al, 2006]. It is interesting to speculate that LPA may activate inside-out integrin signaling by inducing talin conformational change. Alternately, activated FAK (pTyr397) recruits talin to nascent adhesion complexes, independent of direct talin association with β1 integrin in several cell lines including SKOV-3-ip cells [Lawson et al, 2012]. LPA treatment leads to autophosphorylation of FAK (**Fig. 5.4A**); it could be hypothesized that this autophosphorylation event promotes talin recruitment to the integrin cytoplasmic tail, subsequently leading to integrin activation. The G protein subunit $G\alpha_{13}$ has been shown to bind the cytoplasmic tail of integrin αIIbβ3 mediating thrombin-initiated $\alpha_{IIb}\beta_3$ ligation of fibrinogen/fibrin in platelets [Gong et al 2010]. As LPA_2 couples to $G\alpha_{12/13}$ proteins [Choi et al, 2010], it would be interesting to evaluate co-localization of $G\alpha_{13}$ and β1 integrins in ovarian carcinomas following LPA stimulation.

Epithelial ovarian carcinoma (EOC) cells arise from the ovary surface epithelium, forming metastatic foci throughout the peritoneal cavity [Burleson et al, 2004].

EOC cells are rarely found in blood and lymph vasculature, but are found as free-floating cells and multicellular aggregates (MCAs) in the ascites fluid [Naora and Montell, 2005]. These findings support the concept that EOC cells must gain anoikis-resistance during the metastatic cascade. Data presented here show that LPA treatment also decreases programmed cell death evident by decreased caspase-8 and caspsase-9 activity. It is interesting to speculate that one mechanism of metastasizing ovarian tumor MCAs escape from anoikis may be inside-out integrin signaling induced by LPA. LPA induced activating phosphorylation of FAK (tyrosine residues 397 and 576), which is of interest since previous data has shown that phosphorylation and activation of FAK is required for escape from anchorage-dependent programmed cell death [Gilmore, 2005]. It should also be noted that LPA stimulates fibronectin (*FN1*) gene expression, and may potentiate deposition of the matrix component abrogating anchorage loss-mediated cell signaling [Murph et al, 2009].

This work does not address specific signaling pathways by which anoikis is abrogated by LPA stimulation, but data in the literature allows for some speculation. Anti-apoptotic LPA signaling is mediated by phosphatidylinositol 3-kinase (PI3-K) activation in Schwann cells [Weiner et al, 1999], macrophages [Koh et al, 1998] and renal proximal tubular cells [Levine et al, 1997]. LPA also stimulates PI3-K phosphorylation in ovarian cancer cells [Estrella et al, 2007] leading to upregulation of interleukin-6 (IL-6) expression and possibly protection from anoikis via autocrine interleukin signaling [Chou et al, 2005]. LPA is a well-

known transactivator of the epidermal growth factor receptor (EGFR), and EGFR is abberantly overexpressed in ovarian carcinoma [Lafky et al, 2008; Hudson et al, 2009]. Further, activation of EGFR has been associated with conferral of anoikis protection in a number of cell types [Reginato et al, 2003; Jost et al, 2001; Demers 2009; Gianconni et al, 2009; Zoppi et al, 2008]. Consistant with these findings, SKOV3 ovarian carcinoma cells overexpressing EGFR survive following matrix detachment and this survival is enhanced by addition of exogenous EGF [He et al, 2010].

In ovarian carcinoma, anoikis-resistance is conferred by overexpression of the mitchondtrial anti-apoptotic effector Bcl-X_L and downregulation of Bcl-X_L using antisense cDNA restores anoikic sensitivity; however, the mechanism of Bcl-X_L downregulation is unclear [Frankel et al, 2001]. Although a direct role for LPA regulation of Bcl-X_L has not been shown, LPA can prevent translocation of the pro-apoptotic effector Bax from the mitochondria to the cytoplasm and may also induce increased expression of another mitochondrial anti-apoptotic protein Bcl-2 [Deng et al, 2003; Goetzl et al, 1999]. These data suggest that LPA may protect disseminating EOC cells by inhibiting programmed cell death-related mitochondrial signaling.

Surprisingly, one study demonstrates overexpression of Edg-2 (LPA_1), an LPA receptor, induces anoikis in ovarian carcinoma cells lines in an LPA-independent manner [Furui et al, 1999]. EOC cells exhibit loss of LPA_1 and LPA_4 expression

and aberrant gain LPA_2 and LPA_3 expression through an unknown mechanism [Choi et al, 2010]. Loss of LPA_1 in EOC could represent pro-metastatic digression from physiological LPA signaling, leading to anomalous MCA survival. Taken together, the findings in this chapter correspond with literature supporting the theory that LPA confers anoikis resistance to free-floating ovarian tumor cells.

5.8 CHAPTER 5 SUMMARY

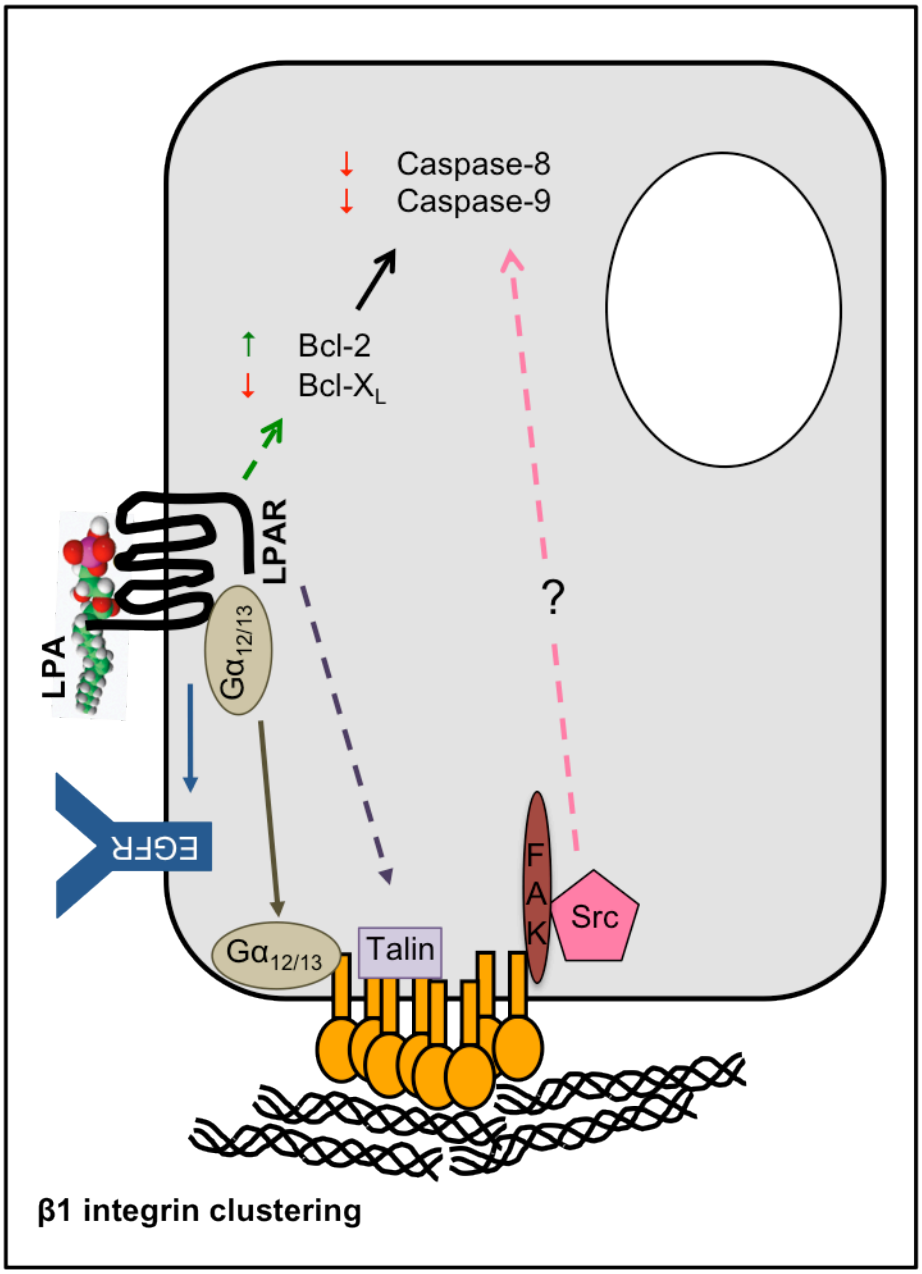


Figure 5.8: Chapter 5 Summary. Lysophosphatidic acid (LPA) potentiates integrin clustering and clustering-dependent signaling. LPA-induced integrin clustering leads to decreased caspase-8 and caspase-9 activity suggesting protection from anchorage-dependent apoptosis (anoikis).

6. DISCUSSION AND CONCLUSIONS

The body of work presented in this dissertation evaluates the effects of microenvironmental factors that regulate ovarian tumor dissemination *in vitro*. Ascites fluid-induced increases in intraperitoneal pressure modulate tumor cell proliferation, protease activity, Wnt/β-catenin target gene expression and cadherin-based cell-cell adhesion (**Fig. 6.1A**). Engagement of integrins, mimicking tumor cell adhesion to the mesothelium and submesothelial matrix, increases Wnt/β-catenin target gene expression (**Fig 6.1B**). Lysophosphatidic acid, expressed in high concentration in ascites fluid, also potentiates β-catenin-dependent Wnt signaling (**Fig. 6.1C**) perhaps in part due to activation of β1 integrin signaling (**Fig 6.1D**). Each microenvironmental factor also increases *WNT5A* gene expression; Wnt5a is overexpressed in the microenvironment, and enhances tumor cell adhesion. Taken together, these data suggest multiple mechanisms for convergent potentiation of metastasis by aspects of the ovarian tumor microenvironment via the Wnt signaling pathway (**Fig. 6.1**).

Activation of Wnt signaling in ovarian carcinoma is histotype dependent [Sarrio et al, 2006]. In contrast to endometrioid ovarian carcinomas, which often harbor

mutations in the β-catenin gene [Palacios and Gamallo, 1998; Saegusa et al, 2001; Schlosshauer et al, 2002; Wu et al, 2001], activating mutations of Wnt signaling pathway components are rare in serous, clear cell and mucinous ovarian carcinomas [Gamallo et al, 1999]. However, nuclear β-catenin has been observed in serous, mucinoid and clear cell ovarian cancers suggesting a mutation-independent mechanism(s) for stimulation/activation (**Fig 3.2, Table 3.1**) [Rask et al, 2003; Marques et al, 2004; Wang et al, 2006; Sagae et al, 1999; Karbova et al, 2002; Lee et al, 2003]. It should be noted that Wnt signaling is not constitutively activated in the majority of individual cells in cases positive for nuclear β-catenin; however current approaches do not enable detection of transient activation of Wnt signaling, such as may occur following initial engagement of tumor cell integrins with sub-mesothelial collagens.

It has been proposed that Wnt/β-catenin target genes can be divided into two groups: a “stemness/proliferation group” that is activated early in tumor progression and an “epithelial-to-mesenchymal transition (EMT)/dissemination group” that is transiently expressed in late stage tumors predominantly at the tumor-host interface [Brabletz et al, 2005]. Disseminating ovarian cancer cells encounter collagen in the peritoneal cavity, thereby suggesting that a temporary EMT or transient loss of junctional E-cadherin may occur to facilitate cancer cell migration and invasion. Integrin-induced E-cadherin downregulation may play an important role in the breaking and reforming of cell-cell junctions during ovarian

cancer cell dissemination, while also contributing to intra-peritoneal anchoring through activation of β-catenin-regulated target genes.

The mechanism of tumor cell migration, whether metastatic success is achieved by single cell migration or collective cell migration, is a source of contention in the literature. Indeed, collective cell migration is a conserved process seen in lower organisms such as Dictyostelium discoideum [Weijer, 2004]. On the contrary, other work suggests that tumor cell motility is facilitated by loss of cell-cell adhesions and gain of fibroblast-like phenotype [Sahai, 2005; Brabletz et al, 2005]. Due to heterogeneity between tumor types, and even within specific tumors, it is more likely that a combination of migratory patterns allow metastatic success. In support of this theory, recent research has found that inhibiting single cell migration differs from cohort migration suggesting migrating cells employ multiple mechanisms (e.g. amoeboid migration, mesenchymal invasion, collective invasion) of motility [Harisi et al, 2009; Friedl and Wolf, 2009]. It could be assumed that the tumor microenvironment, genetic mutations and/or intercellular communication lead to variations in migratory patterns. Further, tumor cell populations may switch between modes of migration to enhance metastatic success [Sahai, 2005; Harisi et al, 2009]. Regardless of metastatic mechanism, shared features include regulation of protease expression and activity to achieve extracellular matrix remodeling and fine-tuned cell-cell and cell-matrix adhesion balancing [Friedl and Gilmour, 2009; Friedl et al, 2004]. Epithelial ovarian carcinoma cells have been shown to metastasize both as

single cells, exhibiting a mesenchymal phenotype, and as multicellular aggregates (MCAs), exhibiting an epithelial-like phenotype depending on adhesion molecule expression and protease profile [Kwon et al, 2011]. Interestingly, the current data supports a theory of multiple metastatic mechanisms, demonstrating differential response to high intraperitoneal fluid pressure and Wnt5a signaling depending on cadherin expression. Moreover, lysophosphatidic acid (LPA) induces protease activity characteristic of both metastasis mechanisms. The ovarian tumor microenvironmental factors evaluated here (integrin engagement, altered intraperitoneal mechanics and LPA expression) upregulate Wnt/β-catenin target genes in both the "stemness/proliferation group" (*WNT5A*, *LRP6*) and the "epithelial-to-mesenchymal transition (EMT)/dissemination group" (*WNT5A*, *VIM*, *COX2*, *PLAUR*, *MMP9*, *SNAI1*), suggesting Wnt/β-catenin signaling and subsequent target gene expression regulates phenotypic plasticity and may direct migration/invasion.

Ascites accumulation and increased intraperitoneal pressure are chronic symptoms of ovarian cancer. The work presented here is a simple evaluation of the effects of short-term (8 hours) altered mechanobiology on ovarian tumor cell pathobiology. Technical issues in the current model prevent prolonged study of increased fluid force, including changes in barometric pressure and appropriate sealing of the cylindrical pressure vessels. Further, the Instrom 8215®, a servohydraulic testing system, is engineered to produce up to hundreds of

pounds of force and may have standard error that is greater than the force being investigated (0.5 pounds per square inch). Future studies will utilize a servohydraulic testing system more suited for mimicry of intraperitoneal pressure operated by a computer-based program [Marszow and Wagner, unpublished work], which will continuously apply pressure and monitor the study so that fluctuations can be observed and corrected. Based on observations of cell viability while cultured in the polystyrene bags [Burkhalter and Stack, unpublished observations], these modifications to the current protocol will allow study of cells cultured under increased pressure for up to 72 hours. Although MCAs in this study were cultured in an adhesion-independent manner, lack of adherent controls prevents disentanglement of shear stress-induced versus fluid pressure-induced genotypic and phenotypic alterations. While the cylindrical vessel model not well-suited for this purpose, the Flexercell ® apparatus may offer a mechanism of studying cell cultured as two-dimensional (2D) monolayers and as MCAs so that consequences of shear stress exposure can be independently studied. Confluent cells or MCAs in suspension can be seeded in BioPress ® culture dishes, then maintained under static or cyclical compressive force up to 72 hours.

Attempts to evaluate subcellular protein localization by immunofluorescent staining were unsuccessful, in part due to the three-dimensional (3D) nature of multicellular aggregates. For instance, modifications of surface/plasma membrane-expressed proteins were difficult to appreciate in these experiments

were difficult to interpret since 3D MCA cytocentrifugation onto 2D coverslips compressed the MCAs producing artifact visible by microscopy that confounded data analysis. Three-dimensional MCAs also prevented analysis of surface-expressed proteins by flow cytometry, as success of fluidic methods is contingent upon laser evaluation of single cells. Current work seeks to circumvent this obstacle by quick-freezing MCAs in liquid Nitrogen, then sectioning (10 μM) aggregates and fixing sections on glass slides for immunohistochemical evaluation of protein expression, activation (e.g. utilizing phosphorylation status-specific or conformation-specific antibodies) and subcellular distribution. These techniques will allow elucidation of the specific biomechanical 'sensors' expressed and activated in ovarian carcinoma cell lines, and the signaling pathways subsequently activated in response to increased fluid pressure.

Each of the microenvironmental factors evaluated in this work upregulate Wnt/β-catenin target genes; however, the β-catenin/Tcf/Lef transcriptome has over 60 gene targets in humans that are relevant to ovarian carcinoma such as *EGFR*, *CD44*, *TWIST1* and *FGF9* [Nusse, 2010]. Gene expression profilers (RT^2 Profiler, Human Targets of Wnt, SA Biosciences) are currently being used to screen for additional Wnt/β-catenin target genes modulated by microenvrionmental mechanical force, LPA signaling and integrin aggregation. Experiments investigating changes in MMP-9, Vimentin, LRP6, uPAR, and Cox-2 protein expression in response to the aforementioned environmental factors are also underway. Exogenous Wnt5a mediates cell adhesion in ovarian carcinoma

cell lines, but the receptor(s) through which its signaling pathway(s) are activated remain unclear. Future work will include profiling ovarian carcinoma cell lines for expression of other Wnt ligands, Frizzled receptors, LRP co-receptors and the alternate Wnt receptors Ryk and Ror. Understanding receptor context(s) in ovarian tumors will allow formation of hypotheses of specific signaling pathway (β-catenin-dependent, β-catenin-independent, etc.) activation. It will also be interesting to assess the functional consequences of aberrant expression and/or pharmacologic inhibition of Wnt5a, and perhaps other unidentified relevant Wnt ligands, in *in vivo* studies.

Currently, a number of therapeutic agents that target Wnt signaling, both loss of function and gain of function alterations, are being investigated in the treatment of cancer, bone disease, cardiac and vascular disease and arthritis [Takahashi-Yanaga and Sasaguri, 2007; Luo et al, 2007]. The anti-Wnt signaling therapeutics are designed to target the extracellular, cytoplasmic, nuclear and pathway crosstalk levels of Wnt signaling. Signaling at all levels can be targeted through downregulation of Wnt ligand expression using antisense molecules, RNAi-based strategy and neutralizing antisense monoclonal antibodies. Interestingly, the non-steroidal anti-inflammatory drug (NSAID) COX-2 inhibitor, celecoxib, has been shown to inhibit Wnt/β-catenin signaling in colorectal tumors. In addition to small molecules, biotherapeutic agents are under development to target the Wnt/β-catenin signaling pathway [Zhang et al, 2010; Luu et al, 2004; Lepourcelet et al, 2004]. These developments in drug discovery present an

exciting potential for more efficacious treatments of ovarian cancer. In summary, the project findings described in this dissertation solidify the diverse roles of ovarian tumor microenvironmental factors and implicate Wnt signaling dysregulation as an important factor in epithelial ovarian carcinoma progression. Understanding the role(s) of Wnt signaling in ovarian cancer may elucidate therapeutic targets allowing for generation of novel, or application of existing, chemotherapeutic and biotherapeutic treatment strategies for better management of the disease.

6.1 PROJECT SUMMARY

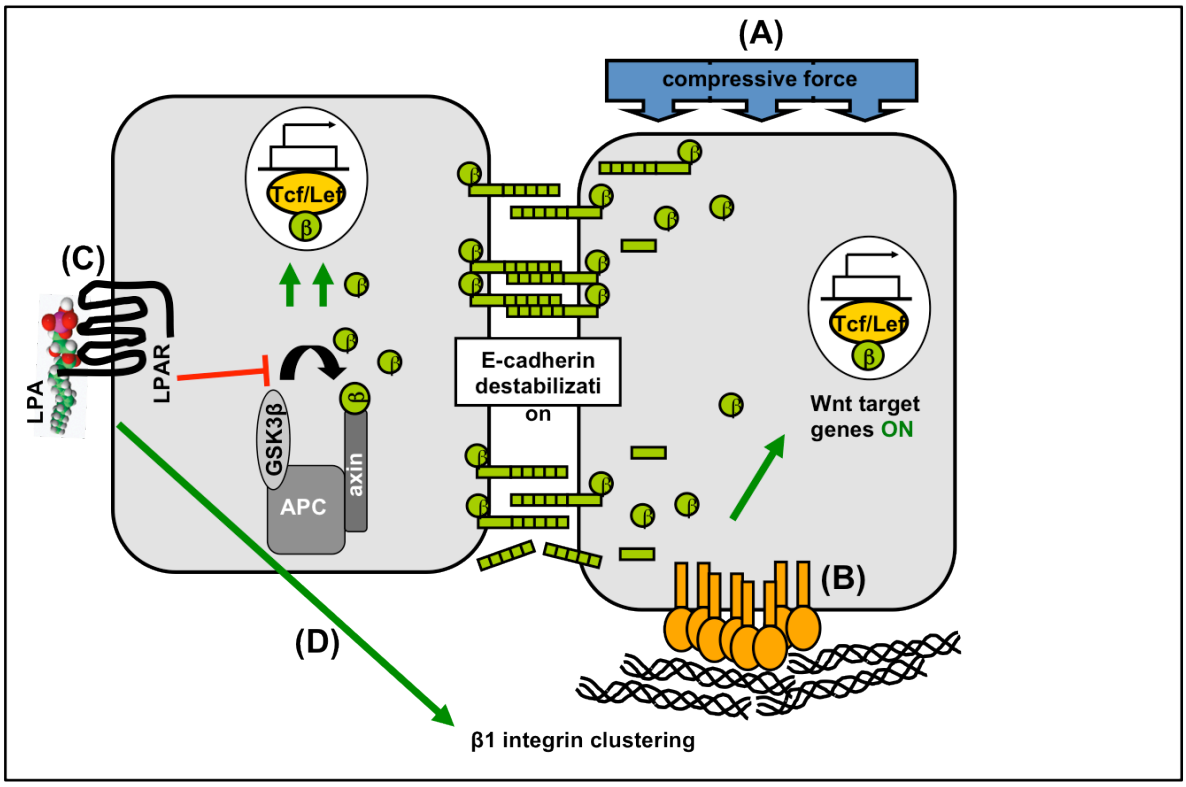


Figure 6.1: Project Summary. A) Compressive force modulates cell proliferation, and cadherin dynamics, and increases expression of Wnt/β-catenin target genes. B) Integrin clustering-induced adherens junction destabilization precedes nuclear translocation of β-catenin and subsequent activation of target gene expression. LPA potentiates ovarian tumor metastasis through activation of β-catenin-dependent signaling, including upregulation of β-catenin target genes, which may secondary to destabilization of cadherin-based cell-cell junctions (C) or LPA-induced integrin aggregation (D).

7. MATERIALS AND METHODOLOGY

I. MODELS

Cell Culture.

OVCA429 and OVCA433 cell lines were chosen for the experiments performed in Chapters 3, 4, and 5 for their high expression of E-cadherin, making them suitable for studies of E-cadherin junction dissolution. Both cell lines were derived from serous-type tumors, which are the most common subtype of ovarian tumors in patients. The OVCA429 and OVCA433 cell lines were generously provided by Dr. Robert Bast (M.D. Anderson Cancer Center, Houston, TX) and were maintained in MEM (Gibco Invitrogen, Carlsbad, CA), 10% fetal bovine serum (Gibco Invitrogen), penicillin/streptomycin (Gibco Invitrogen), amphotericin B, (Cellgro by Mediatech), non-essential amino acids (Cellgro by Mediatech, Herndon, VA), and sodium pyruvate (Cellgro by Mediatech) at 37°C in 5% CO_2.

DOV13 and SKOV3ip cells were used for studies in Chapter 2 for their expression of N-cadherin. As mechanisms of mechanical regulation of ovarian tumor progression are unknown, tumor cells expressing E-cadherin and N-cadherin were evaluated. DOV13 cells were generously provided by Dr. Robert Bast (M.D. Anderson Cancer Center, Houston, TX) and were maintained in MEM (Gibco Invitrogen, Carlsbad, CA), 10% fetal bovine serum (Gibco Invitrogen), penicillin/streptomycin (Gibco Invitrogen), amphotericin B (Cellgro by Mediatech), non-essential amino acids (Cellgro by Mediatech), sodium pyruvate (Cellgro by Mediatech), and insulin from bovine pancreas (10 mg/L) (Sigma-Aldrich, St. Louis, MO) at 37°C in 5% CO_2. SKOV3ip cells were generously provided by Dr. Laurie Hudson, (University of New Mexico, Albuquerque, NM), and maintained in RPMI (Gibco Invitrogen), 10% fetal bovine serum (Gibco Invitrogen), penicillin/streptomycin (Gibco Invitrogen), sodium pyruvate (Cellgro by Mediatech), L-glutamine (Gibco Invitrogen), non-essential amino acids (Cellgro by Mediatech), amphotericin B (Cellgro by Mediatech), HEPES (Gibco Invitrogen), and hygromycin B (Invitrogen) at 37°C in 5% CO_2.

Immunohistochemistry (β-catenin).

Immunohistochemistry analysis was performed on a human tumor tissue microarray prepared at the Robert H. Lurie Comprehensive Cancer Center at Northwestern University.

Ascites Samples.

All ascites samples were collected from patients at Prentice Women's Hospital, in collaboration with Dr. David Fishman and Dr. M. Sharon Stack at Northwestern University with institutional review board consent. Ascites were collected under sterile conditions, and frozen for storage at -20°C. Classification of tumors associated with each ascites sample can be found in APPENDIX 1.

II. MATERIALS

Antibodies.

Mouse anti-human integrin β1 (clone P5D2) and anti-human integrin β1 (clone 21C8) were purchased from Chemicon International (Temecula, CA). Mouse anti-active β-catenin (clone 8E7) recognizes b-catenin that is de-phosphorylated on Ser37 and/or Thr41 and was purchased from Upstate Biotechnology (Lake Placid, NY). Purified mouse anti-β-catenin monoclonal antibody, mouse anti-E-cadherin (clone 36/E-cadherin), anti-active β1 integrin (clone HUTS21), and anti-Hsp90 were purchased from BD Transduction Laboratories (San Jose, CA). Anti-HDAC1 was purchased from Thermo Fisher Scientific (Rockford, IL). Anti-PCNA was purchase from Dako (Glostrup, Denmark). Mouse anti-E-cadherin (clone HECD-1) was purchased from Zymed (San Francisco, CA). Polyclonal mouse anti-IgG, peroxidase-conjugated anti-mouse IgG and peroxidase-conjugated anti-Rabbit IgG were purchased from Sigma-Aldrich (St. Louis, MO). Alexa Fluor 488 goat anti-mouse IgG (H+L) was purchased from Molecular Probes (Eugene, OR). Rat anti-Tcf was purchased from Kamiya Biomedical (Tukwila, WA). Function-blocking mouse anti-MMP9 was purchased from Calbiochem (Darmstadt, Germany).

Other Materials.

Pressure vessels were purchased from Parr Instrument (Moline, IL). Lyophilized Human recombinant Wnt5a-CF (carrier-free) was purchased from R&D Systems (Minneapolis, MN) and resuspended at 1mg/ml in PBS. TOPflash (TCF Reporter Plasmid) and FOPflash (TCF Mutant Reporter Plasmid) were generously provided by Dr. Hans Clevers (Hubrecht Laboratory and Utrecht University, Utrecht, the Netherlands). The Renilla luciferase vector, pRL-CMV, was purchased from Promega (Madison, WI). Diff Quik Stain Kit was purchased from Siemens (Newwark, DE) and used according to manufacturer's instructions. Soybean Trypsin Inhibitor (STI), Val-Leu-Lys-para-nitroanilide (VLK-pNA) and protein A/G beads were purchased from Sigma-Aldrich (St. Louis, MO). The STI was reconstituted at 0.25 mg/ml. Dual Luciferase Reporter Assay System, Caspase-8 Assay and Caspase-9 Assay were purchased from Promega (Madison, WI). Ki16425 (LPA receptor inhibitor) and lyophilized lysophosphatidic acid (LPA) were purchased from Cayman Chemical (Ann Arbor, MI). LPA was prepared for use by dehydrating the lyophilized protein under a tissue culture hood, on ice, overnight. Protein was resuspended in 1% BSA in PBS at a final concentration of 2 mM, allowed to dissolve on a rotator at 4°C overnight, then aliquoted. Aliquots in use were stored at -20°C, and at -80°C for long-term storage. Dispase in DMEM/F12 was purchased from Stem Cell Technologies (Vancouver, British Columbia, Canada). Purified Collagen I was purchased form BD Biosciences (San Jose, CA). Polystyrene beads (3 μM) were purchased as a 2.5% solids (w/v) aqueous suspension from Polysciences, Inc. (Warrington, PA).

Plasminogen was purified by affinity chromatography from human plasma as previously described [Ghosh et al, 2000]. TriZol reagent was purchased from Life Technologies (Carlsbad, CA). Sterile low-density virgin polyethylene bags were purchased from Fisher Scientific, Inc (Hampton, New Hampshire). Gelatin was purchased as a powder from Bio-Rad, Inc (Hercules, CA) and resuspended at 3% (w/v) in PBS.

III. EXPERIMENTAL METHODS

Pressure Chamber: High Fluid Pressure Culture.

To mimic increased peritoneal fluid pressure [Henrickson et al, 1980; Gotlieb et al, 1998], ovarian carcinoma cells were cultured as multicellular aggregates under conditions that mimic the peritoneal cavity as a fluid-filled sac. Culture in stainless steel cylindrical pressure vessels was adapted from a previous study [Wagner et al, 2008; Smith et al, 1996]. The cylindrical pressure vessel had an inside diameter of 9.53 cm and was 12.9 cm deep, with a total volume 920 ml (Parr Instrument, Moline, IL). Twelve milliliters of cell suspension (2×10^6 cells/ml) were seeded into sterile low-density virgin polyethylene bags (Fisher Scientific, Hampton, New Hampshire) and sealed using a bag sealer. Cells were allowed to form multicellular aggregates overnight, then transferred to pre-heated pressure vessels. All components of the pressure vessels were pre-heated to 37°C in an incubator prior to start of assay, and temperature was maintained at 37°C during the assay in an incubator. Pressure was conveyed (0.5 pounds per square inch = 25 mmHg) to the test vessel using the Instron 8215, a servohydraulic testing system, via displacement of water in a medium duty hydraulic pump (PHD, Fort Wayne, IN) connected to the pressure vessel with stainless steel tubing; two valves were closed to contain the fluid pressure. The

control pressure vessel was sealed and incubated along-side the test pressure vessel at atmospheric (room) pressure. After 8 hours, bags were removed from the vessels and cells were harvested by decanting cell suspension into 15 ml conical tubes. Suspensions were processed based on downstream assay application.

Hanging Drop Multicelullar Aggregate (MCA) Formation.

MCAs were generated using a modification of the hanging drop method as previously described [Kelm et al, 2002]. Briefly, cell monolayers were trypsinized and 5 x 10^4 cells were resuspended in 20 µl media supplemented with 10% FBS, and then placed on the cover of a 150-mm tissue culture plate. The cover was inverted over a plate that contained 20 ml of water to prevent dehydration of the hanging droplet. MCA formation was monitored after 24-48 h.

Cell counting.

Multi-cellular aggregates were dispersed by incubation in 1 ml 0.25% trypsin for 10 minutes. Suspensions were diluted in 9 mls phosphate-buffered saline, then counted using a Z2 Coulter Particle Count and Size Analyzer (Beckman Coulter, Brea, CA). All cell counting data was confirmed by hemacytology. Assay was

performed in triplicate for each cell line, and P-value was calculated by paired Student's t-test.

Whole cell lysates.

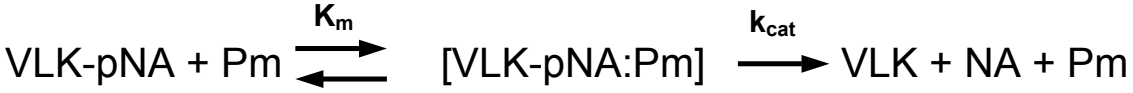
Whole cell lysates were collected by washing cells (two-dimensional cultures or multicellular aggregate pellets) twice in phosphate-buffered saline. Cells were resuspended in 600 µl modified RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA), then passed through a 26 ½ gauge syringe 6 times. Protein concentration was measured using a detergent-compatible protein assay kit (Bio-Rad, Hercules, CA).

Colorimetric Plasminogen Assay.

Urinary-type plasminogen activator (uPA) activity was assessed by colorimetric plasminogen assay, as described in Ghosh et al, 2006. Conditioned medium was collected following cell culture, the centrifuged to remove any particulate. Samples were stored on ice and assayed immediately. Twenty microliters of conditioned medium was added to 20 mM HEPES, pH 7.4, 3 µM plasminogen (Pg), and 3 mM Val-Leu-Lys-para-nitroanilide (VLK-pNA) in a 96 well plate. Plate is placed into a pre-heated (37°C) microtiter plate reader, and monitored by

absorbance at 405 nm. uPA activates Pg, generating the active enzyme Plasmin (Pm):

Pm cleaves VLK-pNA:



The resulting nitrophenolate anion (NA) absorbs at 405nm, and its generation serves as a measure of activity (Wohl et al, 1980).

Western Blotting.

Samples were loaded onto SDS-polyacrylamide gels, electrophoresed, and then transferred onto polyvinylidene fluoride (PVDF) microporous membranes (Millipore). After blocking non-specific binding to membranes in 3% BSA in TBST for 1 hour at room temperature, membranes were incubated with primary antibodies for 3 hours at room temperature or overnight at 4°C, and then with HRP-conjugated secondary antibodies. Immunoreactivity was determined by SuperSignal West Dura Extended Duration Substrate kit (Fisher Scientific). Western blots were quantified using Multigauge v.2 (FUJIFILM, Tokyo, Japan). P-values were determined using the T-test function (two sample, unequal

variance, one tailed distribution) on Excel (Microsoft Corporation, Redmond, WA). PVDF membranes were stripped of antibody by washing in a stripping buffer (50 mM Tris, pH 6.8, 1% SDS, 150 mM NaCl, 100 mM β-mercaptoethanol, 0.02% sodium azide) then re-probed for appropriate assay control(s).

Quantitative Real-Time RT-PCR.

Total RNA was extracted from treated and control cells using TRizol reagent (Life Technologies, San Diego, CA) according to manufacturer's instructions. cDNA was synthesized from 1 μg RNA using the RT^2 First Strand cDNA Synthesis Kit (SA Biosciences, Frederick, MD). Amplification was performed during using iCycler (Bio-Rad, Hercules, CA) for 40 cycles, with each cycle consisting of 15 seconds denaturation at 95.0°C followed by 1 minute of annealing at 60.0°C. SYBR Green Master Mix and primer sets for *RAC1*, *VIM*, *LRP6*, and *WNT5A* were purchased from SA Biosciences (Frederick, MD). GAPDH was used as an internal control in each reaction. The RT2 Profiler PCR Arrays, Wnt Signaling Pathway (PAHS-043A) and Epithelial-to-Mesenchymal Transition (PAHS-090A), were purchased from SA Biosciences (Frederick, MD) and used according to manufacturer's instructions.

Immunohistochemistry.

Tumor specimens were cut 3 to 5 microns thick (1 mm in diameter) and deparrafinized. Antigen retrieval was accomplished by heat induction at 99°C in an antigen retrieval solution (10 mM Tris, 1mM EDTA, pH 9.0) for approximately 1 hour [Baker and van den Born, 2008]. Immunohistochemical staining with anti-β-catenin (BD Transduction Laboratories; 1:50) was performed according to standard procedures and as previously described [Symowicz et al, 2007].

Cell-Matrix Adhesion Assay.

Adhesion plates were prepared by incubation with 10 µg/ml Collagen I (BD Biosciences, San Jose, CA) in 0.1 M sodium carbonate, pH 9.6 at 37°C for two hours. Plates were air-dried, and non-specific binding was blocked by incubation in 3% BSA in serum-free medium for 1 hour at 37°C. Cells were prepared by trypsinization; trypsin was neutralized by addition of 1:1 soybean trypsin inhibitor. After centrifugation and resuspension in serum-free medium, 1 x 10^5 cells were added to each well, and allowed to adhere for 30 – 90 minutes, depending on cell line. Adhesion assay was stopped using the Diff-Quik kit (Siemens, Newark, DE).

Human Wnt5a ELISA.

Wnt5a ELISA was purchased from USCNK Life Sciences Inc. (Wuhan, P. R. China), and performed according to manufacturer's instructions. Fifty micrograms of each sample (ascites or tumor cell line lysate) was added per assay (well). Description of ascites samples can be found in APPENDIX 1.

Dispase-Based Dissociation Assay.

Dispase-based dissociation assay was performed as previously described (Ghosh et al, 2006; Munshi et al, 2006). Briefly, cells were subcultured (triplicate per condition) in 60 millimeter dishes to 80% confluence in MEM. Cultures were washed twice in PBS before being incubated with 2 ml dispase in DMEM/F12 (Stem Cell Technologies, Vancouver, British Columbia, Canada) until the cell monolayer detached from the culture plate. Subsequently, the detached cell monolayer was washed in PBS, transferred to a 15 ml conical tube and subjected to 50 inversion cycles on a bench-top rocker. Any cell fragments were placed into tissue culture dishes and counted (regardless of cell fragment size) by light microscopy. The assay was repeated twice and analyzed statistically by Student's t test.

Immunoprecipitation.

The β-catenin/Tcf co-immunoprecipitation protocol was adopted from chromatin immunoprecipitation protocols previously described (Chamarro et al, 2005 and

Lowry et al, 2005). Cells were grown to 80% confluence, then serum-starved overnight. Appropriate samples were pre-treated with 40 μM Ki16425 (LPA receptor inhibitor, Cayman Chemical, Ann Arbor, MI) or DMSO control, followed by addition of 40 μM LPA for 2 hours. Non-treated cells, cells treated with Ki16425 alone, and cells treated with 40 μM LiCL served as controls. Cells were collected in a hypotonic lysis buffer (20 mM HEPES, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA), incubated on ice for 20 minutes, then centrifuged at 13,000 rpm for 10 minutes at 4°C. Nuclei isolated from the previous step were disrupted by resuspension in a 'breaking' buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl, 1% SDS, 2% triton x-100), then passed through a 26 ½ gauge syringe 8 times. The suspension is centrifuged at 13,000 rpm for 10 minutes at 4°C, diluted in 1 ml triton buffer (50 mM Tris-Hcl, pH 8.0, 1 mM EDTA, 150 mM NaCl, 0.1% triton x-100) and cleared of non-specific binding by incubation with 20 μl protein A/G beads (Sigma-Aldrich, St. Louis, MO) at 4°C overnight. After centrifugation and collection of supernatant, protein concentration is measured using a kit (Bio-Rad). Five hundred micrograms total protein is added to 1 ml triton buffer, 20 μl protein A/G beads and 5 μg anti β-catenin antibody, then incubated on a rotator overnight at 4°C. Beads are washed 5 times in triton buffer, and then resuspended in 2X sample buffer. Samples are boiled, then analyzed for Tcf expression by Western blot as described above. Antibody was removed from the PVDF membrane by washing in stripping buffer (50 mM Tris, pH 6.8, 1% SDS, 150 mM NaCl, 100 mM β-

mercaptoethanol, 0.02% sodium azide) then re-probed for β-catenin as an assay control.

β1 Integrin Crosslinking.

In order to evaluate membrane localization of β1 integrins as individual heterodimers or as clusters, a crosslinking mechanism was utilized to capture integrin distribution following LPA treatment. Analysis of β1 integrin clustering was performed as described previously (Gilcrease et al, 2004). Cells were serum-starved overnight, trypsinized and resuspended in fresh serum-free medium in 1.5 ml Eppendorf tubes. Suspensions were incubated with 40 μM LPA for 40 minutes at room temperature. Control cells were incubated with 1% BSA in PBS (LPA vehicle). In order to crosslink surface expressed β1 integrins, anti-β1 integrin (MAB1959) was added for a 40-minute incubation on ice. Cells were washed, resuspended in serum-free medium, then incubated with polyclonal anti-mouse IgG (Chemicon) at 37°C for 30 minutes. Cell suspensions were affixed to glass 22-mm^2 coverslips by cytology centrifugation using a Cytopro 7620 Centrifuge (Wescor, Logan, UT) with LO acceleration for 2000 rpm for 10 minutes. Clustering was analyzed by immunofluorescent staining (primary antibody: anti-β1 integrin (MAB2250); secondary antibody: anti-mouse IgG – Alexa488), as described below. Fluorescent data was quantified by counting the number of cells with punctate staining patterns indicating clusters, compared with

the total number of slides in a given field (Nikon Microphot FXA, 40X magnification).

Immunofluorescence.

Cells were plated on 22-mm^2 glass coverslips coated by passive adsorption with type I collagen (from rat tail, BD Biosciences, San Jose, CA) and placed in 6 well tissue culture plates for up to 5 days. Control cells were plated on uncoated glass coverslips. Cells were gently washed in phosphate buffered saline (PBS), fixed in paraformaldehyde (4%) at room temperature, washed in PBS, and blocked in PBS/1% bovine serum albumin (BSA) followed by the addition of primary antibody diluted in PBS/1% BSA at 37°C. After two washes in PBS, coverslips were incubated with Alexa Fluor 488 goat anti-mouse IgG (1:500 dilution in PBS/1% BSA) in the dark at room temperature. Coverslips were washed in PBS twice and in distilled water once, fixed using gelvatol, and visualized using fluorescence microscopy (Nikon Microphot FXA).

Cell Fractionation.

Cells were fractionated following a 90-minute incubation with bead-immobilized β1 integrin antibody (control cells were incubated with bead-immobilized IgG antibody) as previously described (Choudhury and Shukla, 2008). Briefly, cells

were washed twice with cold PBS, and lysed with a cold hypotonic lysis buffer (10.0 mM NaCl, 20.0 mM HEPES pH 7.9, 1.0 mM EDTA, 2.0 mM $MgCl_2$, 20.0 mM β-glycerophosphate, 1.0 mM Na_3VO_4, 1.0 mM PMSF, 1.0 mM DTT, 200 mM sucrose, 0.5% NP-40, and 10 μg/mL of each aprotinin, pepstatin A and leupeptin). Lysate was collected by scraping, passed through a 26G syringe and centrifuged at 16,000 × *g* for 1 minutes at 4°C after a 10 minute incubation on ice. The cytoplasmic fraction was collected (supernatant), and the pellet was washed twice with hypotonic lysis buffer before treatment with nuclear extraction buffer (420.0 mM NaCl, 20.0 mM HEPES pH 7.9, 1.0 mM EDTA, 2.0 mM $MgCl_2$, 20.0 mM β-glycerophosphate, 1.0 mM Na_3VO_4, 1.0 mM PMSF, 1.0 mM DTT, 25% glycerol and 10 μg/mL of each aprotinin, pepstatin A and leupeptin). Following a 10-15 minute incubation and 5-minute centrifugation (16,000 × *g* at 4°C), the nuclear fraction was collected. Protein concentration was measured using a detergent-compatible protein assay kit (Bio-Rad, Hercules, CA). Western blot analysis was performed as previously described.

Flow Cytometry Analysis.

Cells were serum-starved overnight, followed by pretreatment of appropriate samples with the LPA receptor inhibitor, Ki16425 (Cayman Chemical, Ann Arbor, MI) for 30 minutes at 37C, and LPA treatment for 2 hours at 37C. Cells were trypsinized, resuspended in serum-free medium then incubated with anti-active β1 integrin (HUTS21 clone, BD Trans Labs, San Jose, CA) for 1 hour. Following

three washings in PBS, cells were incubated, protected from light, with anti-mouse IgG-Alexa488 for 30 minutes at ambient temperature. Excess antibody was removed by washing, cells were resuspended in PBS, then analyzed with the CyAn ADP Analyzer (Beckman Coulter, Brea, CA). All assays were performed three times.

Caspase Activation Assay.

The luminescence-based caspase-8 and caspase-9 activation assays were purchased from Promega (Madison, WI). Cells were seeded by hang-drop method on 150-mm dishes (25 µl drops; 2 dishes per condition), and multicellular aggregates were allowed to form overnight. Multicellular aggregates were collected, then resuspended in serum-free medium. Cells were transferred to 0.5% agarose-coated 96-well plates in serum-free medium overnight, then treated with LPA or 1% BSA in PBS (control) for 24 hours. Cells were lysed and prepared for caspase-8 and -9 assays following manufacturer's instructions, then plates were read on a microtiter plater reader. Individual experiments were performed in biological triplicates; all assays were performed three times. P-values were obtained using Excel, by unpaired Student's t-test.

Antibody-Coated Beads.

Antibodies to β1 integrin (clone PD52) and control IgG were passively absorbed onto polybead amino 3.0-micron microspheres (2.59% solids latex; Polysciences, Inc. Warrington, PA) based on manufacturer's instructions and as previously described (Ghosh et al, 2006). Briefly, bead slurry was washed three times in phosphate-buffered saline. Bead surface was prepared by 4-6 hour incubation with 8% glutaraldehyde, following by adsorption of desired antibody during overnight incubation on a bench-top rotator. Finally, beads were blocked in ethanolamine, then BSA in PBS before resuspension in manufacturer-supplied storage buffer for use.

Isolation and Immunofluorescence Analysis of Nuclei.

Cells treated with either bead-immobilized IgG antibody (control) or bead immobilized β1 integrin antibody were lysed with hypotonic lysis buffer (above) in the absence of NP-40. Lysate was quickly centrifuged, then spun onto poly-l-lysine-coated coverslips using a Cytopro 7620 Centrifuge (Wescor, Logan, UT) with LO acceleration to 2000 rpm for 10 minutes. Anti-β catenin (total) antibody was purchased from BD Trans Labs (Franklin Lakes, NJ). Active anti-β catenin antibody was purchased from Upstate (Billerica, MA). Each primary antibody was labeled using DyLight Antibody Labeling Kits (Pierce, Rockford, IL) according to manufacturer's instructions. Anti-active β-catenin was conjugated with AlexaFluor649 and anti-β-catenin with AlexaFluor488. Antibodies were purified from ammonium ion-/amine-containing buffer by dialysis, and suspended

in PBS. Immunofluorescence was performed as described above, omitting fluorescent secondary antibody.

TOPflash Assay.

OVCA433 cells were plated at 40-50% confluence in 6 well plates and transiently cotransfected with a Renilla luciferase reporter construct (pRL-CMV) and either the firefly luciferase TOPflash TCF Reporter Plasmid or the FOPflash TCF mutant Reporter Plasmid using Fugene 6 Transfection Reagent according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). Approximately 18 hours after transfection, cells were cultured low calcium (0.1mM $CaCl_2$), serum-containing MEM (S-MEM (Invitrogen) for 1 hour before the addition of latex beads coated with TS2/β1 integrin antibody or control IgG for an additional 30 hours. Cells were then lysed in passive lysis buffer (Promega). Both Renilla and Firefly Luciferase readings were taken on a Veritas Microplate Luminometer (Turner Biosystems, Sunnyvale, CA) using the reagents and protocol provided in the Dual Luciferase Reporter Assay System (Promega). Firefly luciferase readings were first normalized to the reading for the corresponding Renilla luciferase reading to account for transfection efficiency. The adjusted TOPflash reading was then normalized to the corresponding adjusted FOPflash reading to account for background reading of the TOPflash construct.

APPENDICES

1. ASCITES SAMPLES

Lane Number	Stage	Tumor Type
1	Normal	
2	Benign	Non-malignant ascites
3	Benign	Right paratubal cyst
4	Benign	Follicular cysts (BRCA-1 mutation)
5	Benign	Benign (BRCA-1 mutation)
6	Benign	Left ovarian dermoid cyst
7	Benign	OHSS (ovarian hyperstimulation syndrome)
8	Stage I	Clear cell carcinoma
9	Stage I	Papillary serous ovarian carcinoma
10	Stage III	Malignant Mixed Mullerian Tumor (MMMT)
11	Stage III	Ovarian Carcinoma (un-typed)
12	Stage III	Papillary serous ovarian adenocarcinoma
13	Stage III	Adenocarcinoma
14	Stage III	Papillary serous endometrial
15	Stage III	Endometroid adenocarcinoma
16	Stage III	Primary peritoneal/ovarian carcinoma
17	Stage III	Ovarian Carcinoma (un-typed)
18	Stage III	Recurrent ovarian carcinoma (un-typed)
19	Stage III	Malignant Mixed Mullerian Tumor (MMMT)
20	Stage III	Immature terotoma
21	Stage III	Papillary serous ovarian adenocarcinoma
22	Stage III	Poorly differentiated mixed serous adenocarcinoma
23	Stage III	Borderline papillary serous adenocarcinoma
24	Stage III	Adenocarcinoma
25	Stage III	Recurrent papillary serous carcinoma
26	Stage III	Adenocarcinoma
27	Stage III	Endometroid adenocarcinoma
28	Stage III	Adenocarcinoma
29	Stage III	Endometroid adenocarcinoma of right ovary with left

		ovary involvement
30	Stage III	Adenocarcinoma
31	Stage IV	Ovarian Carcinoma (un-typed)
32	Stage IV	Adenocarcinoma
33	Stage IV	Adenocarcinoma
34	Stage IV	Metastatic adenocarcinoma
35	Stage IV	Ovarian Carcinoma (un-typed), with liver mets
36	Stage IV	Papillary serous ovarian adenocarcinoma
37	Stage IV	Adenocarcinoma
38	Stage IV	Endometrial clear cell carcinoma
39	Stage IV	Unknown
40	Stage IV	Endometroid adenocarcinoma

2. WNT SIGNALING QRT-PCR ARRAY GENE LIST

Symbol	Description	Gene Name
AES	Amino-terminal enhancer of split	AES-1/AES-2
APC	Adenomatous polyposis coli	BTPS2/DP2
AXIN1	Axin 1	AXIN
BCL9	B-cell CLL/lymphoma 9	LGS
BTRC	Beta-transducin repeat containing	BETA-TRCP/FBW1A
FZD5	Frizzled homolog 5 (Drosophila)	C2orf31/DKFZp434E2135
CCND1	Cyclin D1	BCL1/D11S287E
CCND2	Cyclin D2	KIAK0002
CCND3	Cyclin D3	Cyclin D3
CSNK1A1	Casein kinase 1, alpha 1	CK1/HLCDGP1
CSNK1D	Casein kinase 1, delta	HCKID
CSNK1G1	Casein kinase 1, gamma 1	CSNK1G1
CSNK2A1	Casein kinase 2, alpha 1 polypeptide	CK2A1/CKII
CTBP1	C-terminal binding protein 1	BARS
CTBP2	C-terminal binding protein 2	CTBP2
CTNNB1	Catenin (cadherin-associated protein), beta 1, 88kDa	CTNNB/DKFZp686D02253
CTNNBIP1	Catenin, beta interacting protein 1	ICAT
CXXC4	CXXC finger 4	IDAX
DAAM1	Dishevelled associated activator of morphogenesis 1	FLJ41657
DIXDC1	DIX domain containing 1	CCD1
DKK1	Dickkopf homolog 1 (Xenopus laevis)	DKK-1/SK
DVL1	Dishevelled, dsh homolog 1 (Drosophila)	DVL
DVL2	Dishevelled, dsh homolog 2 (Drosophila)	DVL2
EP300	E1A binding protein p300	KAT3B/p300
FBXW11	F-box and WD repeat domain containing 11	BTRC2/BTRCP2
FBXW2	F-box and WD repeat domain containing 2	FBW2/Fwd2
FGF4	Fibroblast growth factor 4	HBGF-4/HST

FOSL1	FOS-like antigen 1	FRA/FRA1
FOXN1	Forkhead box N1	FKHL20/RONU
FRAT1	Frequently rearranged in advanced T-cell lymphomas	FLJ97193
FRZB	Frizzled-related protein	FRE/FRITZ
FSHB	Follicle stimulating hormone, beta polypeptide	FSHB
FZD1	Frizzled homolog 1 (Drosophila)	DKFZp564G072
FZD2	Frizzled homolog 2 (Drosophila)	FZD2
FZD3	Frizzled homolog 3 (Drosophila)	Fz-3/hFz3
FZD4	Frizzled homolog 4 (Drosophila)	CD344/EVR1
FZD6	Frizzled homolog 6 (Drosophila)	Hfz6
FZD7	Frizzled homolog 7 (Drosophila)	FzE3
FZD8	Frizzled homolog 8 (Drosophila)	FZ-8/hFZ8
GSK3A	Glycogen synthase kinase 3 alpha	DKFZp686D0638
GSK3B	Glycogen synthase kinase 3 beta	GSK3
JUN	Jun oncogene	AP-1/AP1
KREMEN1	Kringle containing transmembrane protein 1	KREMEN/KRM1
LEF1	Lymphoid enhancer-binding factor 1	DKFZp586H0919/TCF1ALPHA
LRP5	Low density lipoprotein receptor-related protein 5	BMND1/EVR1
LRP6	Low density lipoprotein receptor-related protein 6	ADCAD2
MYC	V-myc myelocytomatosis viral oncogene homolog (avian)	MRTL/bHLHe39
NKD1	Naked cuticle homolog 1 (Drosophila)	Naked1
NLK	Nemo-like kinase	DKFZp761G1211
PITX2	Paired-like homeodomain 2	ARP1/Brx1
PORCN	Porcupine homolog (Drosophila)	DHOF/FODH
PPP2CA	Protein phosphatase 2 (formerly 2A), catalytic subunit, alpha isoform	PP2Ac/PP2CA
PPP2R1A	Protein phosphatase 2 (formerly 2A), regulatory subunit A, alpha isoform	PR65A
PYGO1	Pygopus homolog 1 (Drosophila)	DKFZp547G0910

RHOU	Ras homolog gene family, member U	ARHU/CDC42L1
SENP2	SUMO1/sentrin/SMT3 specific peptidase 2	AXAM2/DKFZp762A2316
SFRP1	Secreted frizzled-related protein 1	FRP/FRP-1
SFRP4	Secreted frizzled-related protein 4	FRP-4/FRPHE
FBXW4	F-box and WD repeat domain containing 4	DAC/FBW4
SLC9A3R1	Solute carrier family 9 (sodium/hydrogen exchanger), member 3 regulator 1	EBP50/NHERF
SOX17	SRY (sex determining region Y)-box 17	FLJ22252
T	T, brachyury homolog (mouse)	TFT
TCF7	Transcription factor 7 (T-cell specific, HMG-box)	TCF-1
TCF7L1	Transcription factor 7-like 1 (T-cell specific, HMG-box)	TCF-3/TCF3
TLE1	Transducin-like enhancer of split 1 (E(sp1) homolog, Drosophila)	ESG/ESG1
TLE2	Transducin-like enhancer of split 2 (E(sp1) homolog, Drosophila)	ESG/ESG2
WIF1	WNT inhibitory factor 1	WIF-1
WISP1	WNT1 inducible signaling pathway protein 1	CCN4/WISP1c
WNT1	Wingless-type MMTV integration site family, member 1	INT1
WNT10A	Wingless-type MMTV integration site family, member 10A	FLJ14301
WNT11	Wingless-type MMTV integration site family, member 11	HWNT11
WNT16	Wingless-type MMTV integration site family, member 16	WNT16
WNT2	Wingless-type MMTV integration site family member 2	INT1L1/IRP
WNT2B	Wingless-type MMTV integration site family, member 2B	WNT13/XWNT2
WNT3	Wingless-type MMTV integration site family, member 3	INT4
WNT3A	Wingless-type MMTV	MGC119418

	integration site family, member 3A	
WNT4	Wingless-type MMTV integration site family, member 4	SERKAL/WNT-4
WNT5A	Wingless-type MMTV integration site family, member 5A	hWNT5A
WNT5B	Wingless-type MMTV integration site family, member 5B	MGC2648
WNT6	Wingless-type MMTV integration site family, member 6	WNT6
WNT7A	Wingless-type MMTV integration site family, member 7A	WNT7A
WNT7B	Wingless-type MMTV integration site family, member 7B	WNT7B
WNT8A	Wingless-type MMTV integration site family, member 8A	WNT8D
WNT9A	Wingless-type MMTV integration site family, member 9A	WNT14
B2M	Beta-2-microglobulin	B2M
HPRT1	Hypoxanthine phosphoribosyltransferase 1	HGPRT/HPRT
RPL13A	Ribosomal protein L13a	RPL13A
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	G3PD/GAPD
ACTB	Actin, beta	PS1TP5BP1
HGDC	Human Genomic DNA Contamination	HIGX1A
RTC	Reverse Transcription Control	RTC
RTC	Reverse Transcription Control	RTC
RTC	Reverse Transcription Control	RTC
PPC	Positive PCR Control	PPC
PPC	Positive PCR Control	PPC
PPC	Positive PCR Control	PPC

3. qRT-PCR PRIMER SETS

Cox2	
Forward	5’-GCCCAGCACTTCACGCATCAG-3’
Reverse	5’-AGACCAGGCACCAGACCAAAGACC-3’
GAPDH	
Forward	5’GAGTCAACGGATTTGGTCGT-3’
Reverse	5’-TTGATTTTGGAGGGATCTCG- 3’
MMP9	
Forward	5’-TGGGGGGCAACTCGGC-3’
Reverse	5’-GGAATGATCTAAGCCCAG-3’
Snai1	
Forward	5’-TTCCAGCAGCCCTACGACCAG-3’
Reverse	5’-CGGACTCTTGGTGCTTGTGGA-3’
uPAR	
Forward	5’-GGTGACGCCTTCAGCATGA-3’
Reverse	5’-CCCACTGCGGTACTGGACAT-3’

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VITA

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