THE ROLE OF AKT1 AND IKKβ IN OVARIAN CANCER
TUMORIGENESIS AND CHEMOTHERAPEUTIC RESISTANCE

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By
Roxana Niculaita
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Dissertation written by

Roxana Niculaita

MD, “Carol Davila” University of Medicine and Pharmacy, Romania, 2000
PhD, Kent State University, 2008

Approved by

_________________________________ Chair, Doctoral Dissertation Committee
Nywana Sizemore, PhD

_________________________________ Members, Doctoral Dissertation Committee
Brent Bruot, PhD

_________________________________
Jennifer Marcinkiewicz, PhD

_________________________________
Gail Fraizer, PhD

_________________________________
John R. D. Stalvey, Ph.D

Accepted by

_________________________________ Director, School of Biomedical Sciences
Robert V. Dorman, Ph.D

_________________________________ Dean, College of Arts and Sciences
John R. D. Stalvey, Ph.D
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DEDICATION

I would like to dedicate this thesis to my husband, Dumitru Adrian, and daughters, Maria and Ioana, who have been the joy of my life and gave me the reason to go on during these past few years. I love you all dearly.

I would also like to dedicate my work to the memory of my mother-in-law.

Florica Niculaita.
First of all, I would like to thank my advisor, Nywana Sizemore, without whom my achievement would not have been possible. Thank you for your excellent scientific guidance, thank you for allowing me to be part of your lab and for training me as a scientist during my graduate career. I am profoundly indebted to you and I will always cherish your friendship.

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ABBREVIATION LIST

\( \alpha = \alpha \)-helical domain.

A2780 AKT1p1 = AKT1 knockdown pool of the A2780 ovarian cancer cell line

A2780 IKK\( \beta \)p1 = IKK\( \beta \) knockdown pool of the A2780 ovarian cancer cell line

aa = amino acid

ACS = American Cancer Society

AFP = alfa fetal protein

AKT = serine-threonine specific protein kinase, aka PKB (protein kinase B)

AKT1, AKT2, and AKT3. = the 3 human isoforms of AKT

APC = adenomatosis polyposis coli

ATCC = American Type Culture Collection

BAD = Bcl-2-associated death promoter protein

BCR = breakpoint cluster region

BOC = breast and ovarian cancer

BRCA1, BRCA2 = breast cancer 1 protein, Breast Cancer Type 2 susceptibility protein

\( \beta \)TrCP = Beta-transducin repeat containing protein

C = control (untreated)

\( ^\circ \text{C} \) = Celsius degree

C2 domain = a protein structural domain involved in targeting proteins to cell membranes
CC = coiled-coil domain, a structural motif in proteins (2-7 alpha-helices are coiled together), part of proteins with important biological functions (transcription factors, oncoproteins)

CCF = Cleveland Clinic Foundation

cIAP = cellular inhibitors of apoptosis

CK1 = Casein kinase 1

Cl = chloride

CO₂ = Carbon dioxide

CPT = Cisplatinum

DNA = Deoxyribonucleic acid

ECM = Extracellular matrix

EDTA = ethylenediaminetetraacetic acid

EMSA = Electrophoretic Mobility Shift Analysis

eNOS = endothelial nitric oxide synthase

FDA = Food And Drug Administration

FH = forkhead transcription factors

FIGO = International Federation of Gynecology and Obstetrics

Frz – Frizzled (Wnt receptor)

G418 = Geneticin

GF = growth factor

GRR = glycine rich region.

GSK3 = Glycogen synthase kinase 3
HBSS = Hank's balanced salt solution
HLH = helix-loop-helix domain
HM = hydrophobic motif
HNPCC = hereditary nonpolyposis colorectal cancer
hTERT = human telomerase reverse transcriptase
IC$_{50}$ = inhibitory dose to achieve 50% cell death
IFN$_{\gamma}$ = interferon gamma
I$\kappa$B = Inhibitor of kappa B proteins
IKK = I$\kappa$B kinase, specifically phosphorylates the inhibitory I$\kappa$B$\alpha$ protein to activate NF-$\kappa$B
IKK$\alpha$ (IKK1) = catalytic subunit of the IKK complex
IKK$\beta$ (IKK2) = catalytic subunit of the IKK complex
IKK$\gamma$ (NEMO) = regulatory subunit of the IKK complex
IL-2 = interleukin 2
iSH2 = inter SH2 domain
KD = kinase domain
L = sample treated with L-cells conditioned media
LEF = Lymphoid enhancer-binding factor
LOH = loss of heterozygosity
LPA = lysophosphatidic acid
LRI = Lerner Research Institute
LZ = leucine zipper domain

xvii
MDM2 = murine double minute oncogene

µl = microliter

µM = micromolar

MMP = Matrix metalloproteinases

MTS = [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt

NBD = NEMO-binding domain

NFκB = nuclear factor-kappa B

nM = nanomolar

P = phosphorylated

pAKT = phosphorylated AKT

PCTX = Paclitaxel

PDK1 = phosphoinositide-dependent kinase 1

PDZ domain = common structural domain of signaling proteins; PDZ = acronym of the first letters of three proteins: post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (DlgA), and zonula occludens-1 protein (zo-1)

PHD = pleckstrin homology domain, a protein domain (120 amino acids) in proteins involved in intracellular signaling

PI3K or PI 3-kinase = Phosphoinositide 3-kinase

PI3KCA = p110α catalytic subunit of PI3K

p IκB = phosphorylated IκB

PIP2 = phosphatidylinositol (4, 5) diphosphate
PIP3 = Phosphatidylinositol (3, 4, 5)-trisphosphate
PRD = proline rich domain
P/S = penicillin/ streptomycin
Pt = Platinum
PTEN = phosphatase and tensin homolog deleted on chromosome ten
RAS = family of proto-oncogenes (small GTP-ases)
Rel HD = Rel Homology Domain
RNA = Ribonucleic acid
RNAi = RNA interference
RPMI = Roswell Park Memorial Institute medium
RTK = receptor tyrosine kinase
SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis,
SH2 = Src homology 2 domain, a protein domain of about 100 amino acid residues
typically binds a phosphorylated tyrosine residue
SH3 = Src homology 3 domain, small protein domain of about 60 amino acid residues
found in proteins that interact with other proteins and mediate formation of protein
complexes (by binding to proline-rich peptides in their respective binding partner)
shRNA = short hairpin RNA
SKOV-3 AKT1p1 = AKT1 knockdown pool of the SKOV-3 ovarian cancer cell line
SKOV-3 IKKβp1 = IKKβ knockdown pool of the SKOV-3 ovarian cancer cell line
SPARC = Secreted protein, acidic, cysteine-rich (osteonectin)
T = sample treated with TNFα
TA, TAD = transactivation domain
TCF = T-cell factor
TGFBI = Transforming growth factor, beta-induced
TNFα = tumor necrosis factor α
TUNEL = Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labeling assay
Ub = Ubiquitinated
UK = United Kingdom
US = United States
VEGF = Vascular endothelial growth factor
W = sample treated with Wnt3a conditioned media
WB = western blot analysis
WBC = White blood cells
WWII = World War II
Z = zinc finger domain
CHAPTER 1

INTRODUCTION

1.1. Introduction to ovarian cancer

Ovarian cancer is the seventh most common cancer and it accounts for approximately 4% of all cancers in women. It is the 4th leading cause of cancer-related death among women in the US. In the year 2007, an estimated 22,430 new ovarian cancer cases were diagnosed in the US and 15,280 patients succumbed to the disease. Even though during the last twenty years the incidence of ovarian cancer has declined, ovarian cancer still has the highest mortality of all cancers of the female reproductive system, reflecting, in part, a lack of early symptoms and proven ovarian cancer screening tests (Decherney et al, Macdonald et al).

Ovarian cancer is more common among women from industrialized countries. It has the highest incidence in western European countries (such as: Sweden, UK etc) (Kantarjian HM et al, 2006). White women have higher incidence and mortality rates than other racial and ethnic groups (U.S. Cancer Statistics Working Group). The risk of getting ovarian cancer for a woman during her entire lifetime is 1.5% (around 1:70) and the chance of dying from ovarian cancer in around 1.0% (American Cancer Society).
Table 1: FIGO Staging System for ovarian cancer (adapted)

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Tumor limited to ovaries</td>
</tr>
<tr>
<td>IA</td>
<td>Tumor limited to one ovary; no ascites, no tumor on the external surface; intact capsule</td>
</tr>
<tr>
<td>IB</td>
<td>Tumor limited to both ovaries; no ascites, no tumor on the external surfaces; intact capsules</td>
</tr>
<tr>
<td>IC</td>
<td>Tumor either stage IA or IB but with tumor on the surface of one or both ovaries; or with capsule ruptured; or with ascites present containing malignant cells or with positive peritoneal washings</td>
</tr>
<tr>
<td>II</td>
<td>Tumor in one/both ovaries + pelvic extension.</td>
</tr>
<tr>
<td>IIA</td>
<td>Extension and/or metastases to the uterus and/or tubes</td>
</tr>
<tr>
<td>IIB</td>
<td>Extension to other pelvic tissues</td>
</tr>
<tr>
<td>IIC</td>
<td>Tumor either stage IIA or IIB with tumor on the surface of one or both ovaries; or with capsule(s) ruptured; or with ascites present containing malignant cells or with positive peritoneal washings</td>
</tr>
<tr>
<td>III</td>
<td>Tumor in one/both ovaries and/or positive retroperitoneal or inguinal nodes. Superficial liver metastasis equals stage III. Tumor is limited to the true pelvis, but with histologically proven malignant extension to small bowel or omentum</td>
</tr>
<tr>
<td>IIIA</td>
<td>Tumor grossly limited to the true pelvis with histologically confirmed implants of abdominal peritoneal surfaces, none exceeding 2 cm in diameter. Nodes negative</td>
</tr>
<tr>
<td>IIIB</td>
<td>Tumor of one or both ovaries with histologically confirmed implants of abdominal peritoneal surfaces, none exceeding 2 cm in diameter. Nodes negative</td>
</tr>
<tr>
<td>IIIC</td>
<td>Abdominal implants more than 2 cm in diameter and/or positive retroperitoneal or inguinal nodes</td>
</tr>
<tr>
<td>IV</td>
<td>Tumor of one/both ovaries + distant metastasis. Parenchymal liver metastasis equals stage IV</td>
</tr>
</tbody>
</table>
Ovarian cancer is a disease that affects women at their peri- and post-menopausal age, with the highest incidence among patients 65 – 74 years old (Decherney et al; Kantarjian HM et al, 2006)

Ovarian cancer is an intra-abdominal process, which in most cases does not produce symptoms until after the cancer has spread beyond the ovary, therefore it is often diagnosed at an advanced stage (FIGO Stage III of IV) (Table 1). Approximately 3 out of 4 of women with ovarian cancer survive 1 year after diagnosis, but less than half survive more than 5 years after diagnosis. Younger women, if diagnosed before the cancer has spread, have a higher survival rate (over 90% at 5 years) (Table 2). However, less that 20% of cases are diagnosed at this early relatively curable stage of disease due to the lack of symptoms, therefore prevention of and better therapy for ovarian cancer remains an important goal for researchers and clinicians alike (American Cancer Society).

Table 2: Five year relative survival rate for ovarian cancer by stage at diagnosis (1996-2003), adapted from ACS, Surveillance Research, 2008

<table>
<thead>
<tr>
<th>Site</th>
<th>All stages (%)</th>
<th>Local (%)</th>
<th>Regional (%)</th>
<th>Distant (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovary</td>
<td>44.9</td>
<td>92.4</td>
<td>71.4</td>
<td>29.8</td>
</tr>
</tbody>
</table>
1.2. Etiology and genetics of ovarian cancer

The etiology of ovarian cancer is not completely understood; continuous uninterrupted ovulation is considered to play a key role in the development of ovarian cancer. It has been observed that the temporary suppression of menstrual function during pregnancy (multiparity), lactation (breast feeding) and oral contraceptive use, acts as a protective factor. Each pregnancy reduces the risk by approximately 15% and the use of oral contraceptives for 5 years or more is associated with a reduction of more than 50% in the risk of epithelial ovarian cancer (Decherney et al, Collaborative Group on Epidemiological Studies on Ovarian Cancer, Lurie et al. 2008, Kiley et al, 2007). It has also been observed that bilateral tubal ligation is a protective factor, most like acting as a barrier against ascending carcinogenic substances (Tworoger et al, 2007; DeCherney et al).

A number of risk factors have been associated with ovarian cancer. These include nulliparity (twice higher than the general population), family history (up to 4 times higher than the general population), use of talc and diets high in saturated animal fats (Kantarjian HM et al, 2006; DeCherney et al; Bandera et al, 2007; Colombo et al, 2006; Cramer et al, 1984; Harlow et al, 1992). Fertility treatments and hormone replacement therapy have been studied but have not shown a consistent and significant effect on ovarian cancer risk (Kantarjian HM et al, 2006).

In about 90% of cases, ovarian cancer occurs sporadically. Approximately 10% of the epithelial ovarian cancer cases have a genetic predisposition. The hereditary ovarian cancer can take 2 forms, either as Lynch II syndrome also known as breast and ovarian
cancer (BOC) syndrome or as hereditary nonpolyposis colorectal cancer (HNPCC) syndrome. The Lynch II syndrome is associated with $BRCA1$ and $BRCA2$ mutations. For women that are carriers of $BRCA1$ mutations, the lifetime risk of developing ovarian cancer is almost 50% for women carrying $BRCA1$ mutations and approximately 25% for those with $BRCA2$ mutations. The HNPCC syndrome consists of familial colon cancer with associated ovarian, endometrial, gastrointestinal or genitourinary malignancies and is caused by mutations in DNA mismatch repair genes. These hereditary forms of ovarian cancer are transmitted vertically, autosomal dominant, and develop about 10 years earlier than the sporadic ovarian cancer. (DeCherney et al; Abelloff et al).

Allelic loss and mutations of the $p53$ tumor suppressor gene are found in more than half of all ovarian cancers but occur mainly in serous and endometrial ovarian cancers (Sanseverino F et al, 2005; Willner J et al, 2007). Over-expression of c-Myc and oncogenic mutations in $K$-ras, $p16$, and $HER2/neu$ occur in epithelial serous ovarian tumors (Sanseverino F et al, 2005). $\beta$-catenin mutations are mainly found in endometroid tumors (Sanseverino F et al, 2005; Willner J et al, 2007). Human epidermal growth factor receptor 2 ($HER-2/neu$ is overexpressed in about 1/3 of ovarian cancers and is associated with poor clinical prognosis and survival (Kantarjian HM et al, 2006). Other molecular mechanisms involved in ovarian cancer development and progression are activation of the phosphatidylinositol 3-kinase 3-kinase (PI3K)/AKT pathway. This can occur through $PI3K$ $p110$ alpha ($PI3CA$) amplifications, phosphatase and tensin homolog deleted on chromosome ten ($PTEN$) mutations (endometrioid tumors), and $AKT2$ amplifications (serous carcinoma) (Sanseverino F et al, 2005; Willner J et al, 2007).
Other tumor suppressor genes are probably involved in ovarian cancer tumorigenesis, as loss of heterozygosity (LOH) studies have identified several chromosomal regions lost in ovarian cancer (Bell DA et al, 2005; Boerboom Det al, 2005; Burgering BM et al, 1995; Chen WS et al 2001; Datta K et al, 1995, Giles RH et al, 2003).

1.3. Classification of ovarian cancer

The ovary can be the primary organ site of cancer or it can be the metastatic site of cancer from other organs.

1. Primary ovarian cancer

There are 3 major forms of primary ovarian cancer depending on the cell type of origin: epithelial neoplasms, germ cell neoplasms and sex cord stromal tumors.

Table 3: Major Histopathologic Categories of Ovarian Cancer (adapted from DeCherney, 2007)

<table>
<thead>
<tr>
<th>Category</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelial</td>
<td>serous mucinous, endometrioid, clear cell, transitional cell, undifferentiated</td>
</tr>
<tr>
<td>Germ Cell</td>
<td>dysgerminoma, endodermal sinus tumor, teratoma, embryonal cell carcinoma, choriocarcinoma, gonadoblastoma, mixed germ cell, polyembryona</td>
</tr>
<tr>
<td>Sex Cord Stromal</td>
<td>granulosa cell tumor, thecoma, Sertoli-Leydig cell</td>
</tr>
<tr>
<td>Neoplasms Metastatic to the Ovary</td>
<td>breast, colon, stomach, endometrium, lymphoma</td>
</tr>
</tbody>
</table>
1.3.1. *Epithelial neoplasms*

Epithelial tumors represent over 60% of all ovarian neoplasms and more than 90% of the malignant ovarian tumors. They occur primarily in adults. They are derived from the ovarian surface mesothelial cells. The major cell types of epithelial ovarian cancer are: serous, mucinous, endometrioid, clear cell, transitional cell and undifferentiated.

**Serous tumors** account for 50 -70% of epithelial ovarian cancers (most common form). They are large tumors, bilateral in about 50% of cases and in over 75% of cases at the time of diagnosis the cancer had already spread outside the ovary.

**Mucinous tumors** account for about 10% of all epithelial ovarian cancers and in about 10 % of cases are bilateral. These tumors are often quite large by the time of diagnosis.

**Endometrioid tumors** represent about 10% of epithelial cancers and they resemble to endometrial adenocarcinoma. It can be bilateral in 30–50% of cases. Approximately 30% of patients will simultaneously develop primary endometrioid tumors of the ovary and primary endometrial carcinoma of the uterus.

**Clear cell carcinomas** of the ovary (called like this due to the resemblance to the “clear cells” seen in renal carcinoma) represent 1 – 5 % of epithelial ovarian cancers. They are smaller in size compared to the serous or mucinous tumors but biologically aggressive; hypercalcemia and hyperpyrexia may be associated with clear cell tumors.

**Transitional cell carcinomas** are rare tumors, representing less than 1% of all epithelial ovarian cancers. They resemble the transitional cell carcinomas of the urinary
bladder. Are often diagnosed at an advanced stage and the prognosis is worse compared
to other types of epithelial ovarian cancer.

*Undifferentiated carcinoma* accounts for 10 % of ovarian epithelial cancers and
is associated with a poor prognosis.

1.3.2. **Germ cell neoplasms**

Germ cell neoplasms consist of a variety of histologically different tumors that
derive from the primitive germ cells of the embryonic gonad. Their incidence peaks in
the second and third decades of life and they are associated with a better prognosis. They
represent the second largest group of ovarian neoplasms (approximately 15%).

Germ cell neoplasms include: dysgerminoma, endodermal sinus tumor (yolk sac
tumor), embryonal cell carcinoma, choriocarcinoma, teratoma, polyembryoma, and
mixed germ cell tumors.

1.3.3. **Sex cord stromal neoplasms**

Sex cord – stromal tumors of the ovary represent about 5% of all ovarian cancers
and they are hormonally active tumors. They are composed of various cell types derived
from gonadal stroma and sex cords. Unlike epithelial ovarian cancers, most of the sex
cord stromal neoplasms are diagnosed at an early stage.

Sex cord stromal tumors include: granulosa cell tumors, thecomas, Sertoli cell
tumors.
II. Ovarian metastatic disease

Ovarian metastases represent around 5% of all ovarian cancers. The ovary is the metastatic site for cancers of the female genital tract (uterus), breast and gastrointestinal tract (colon, pancreas, stomach).

1.4. Diagnosis and treatment of ovarian cancer

Typically, ovarian cancer is a disease that develops silently, without symptoms or warning signs. By the time the disease becomes symptomatic, it has already disseminated outside the ovary. The most common sites of metastasis for ovarian cancer are the peritoneum omentum, pelvic lymph nodes, paraaortic lymph nodes, liver, pleura, lung, and bone (DeCherney et al; Kantarjian HM et al, 2006; Chobanian et al, 2008; American Cancer Society).

Patients with ovarian cancer may have a history of gastrointestinal symptoms: nausea, indigestion, constipation or diarrhea, abdominal pain or bloating which is easily misdiagnosed as far less serious disorders. Abdominal extension is a sign of ascites and advanced disease. Reproductive age patients may present menstrual abnormalities. Vaginal bleeding may occur in patients that have concomitant ovarian cancer and endometrial cancer (Ryerson et al. 2007, BJOG: an International Journal of Obstetrics and Gynaecology, DeCherney et al). Germ cell neoplasms may be associated with increased levels of estrogens or androgens (DeCherney et al; Kantarjian HM et al, 2006).

The prognosis of ovarian cancer is significantly better if the disease is diagnosed at an early stage, before it spreads outside the ovary. The 5-year survival rate for ovarian
cancers diagnosed while the cancer was limited to the ovary is 92.4%, while for the cancers with distant metastases is 29.4% (American Cancer Society, 2008). Mucinous and endometrioid neoplasms have a better prognosis than serous and clear cell carcinomas of the ovary. Germ cell tumors are associated with better 5-year survival rates than any of the epithelial ovarian neoplasms.

The best tumor marker for epithelial ovarian cancer is cancer antigen 125 (CA-125). CA125 is a secreted glycoprotein which can be detected in the serum of the patient, and values above 35IU/ml are considered elevated. Even though CA125 is not specific for ovarian cancer, it is suggestive of an ovarian malignancy in a postmenopausal woman. The diagnosis of ovarian cancer is based on a combination between routine pelvic and abdominal examination, ultrasound and CT scans, laboratory tests (CA125 for epithelial cancer; alpha fetal protein [AFP] for germ cell tumors) (Alletti et al, 2007; Colombo et al, 2006; Guppy et al, 2005; Schorge J et al; DeCherney et al; Walsh et al, 2007).

Treatment of ovarian cancer involves cytoreductive surgery (including hysterectomy, bilateral salpingo-oophorectomy, and omentectomy), chemotherapy (a combination of paclitaxel and cisplatin/ carboplatin), radiotherapy (limited for epithelial ovarian cancer but successfully used for germ cell neoplasms), and alternative therapies including interleukin 2 (IL-2), interferon gamma (IFNγ), and monoclonal antibodies against CA125, HER 2/neu (DeCherney et al; Kantarjian HM et al, 2006; Abeloff D et al, 2004;Colombo et al, 2006; Alletti et al, 2007; Oei et al, 2007, Williams et al, 2007; Markman M, 2008).
1.5. PI3K/AKT/PTEN pathway

The activation of oncogenes and the inactivation of tumor suppressor genes is critical to tumor development and progression.

PTEN (phosphatase and tensin homologue deleted on chromosome ten) // mutated in multiple advanced cancers (MMAC) is a tumor suppressor gene located on chromosome 10 which encodes for a dual specificity phosphatase. PTEN is a 403 amino acid long protein that contains an NH₂-terminal phosphatase domain closely associated with the COOH-terminal C2 domain, both required for enzymatic activity (Fig. 1A). PTEN dephosphorylates the D3 position of the phosphatidylinositol (3, 4, 5) trisphosphate (PIP₃) lipid product of PI3K, antagonizing the PI3K/AKT cell survival pathway (Maehama et al. 1998; Myers et al. 1998; Myers et al. 1997) (Fig.1C).

PI3K is a ubiquitous lipid kinase consisting of a heterodimer formed by an 110kDa catalytic subunit (p110) and an 85kDa regulatory subunit (p85) (Fig. 1B). Upon activation of several growth factor and cytokine receptors, PI3K is recruited to the plasma membrane producing 3' phosphoinositide lipids, which act as second messengers by binding to and activating several cellular target proteins (Burgering et al. 1995; Sizemore et al. 1999).

The p110 PI3K subunit (PI3CA) specifically catalyzes the phosphorylation of the D3 position of phosphatidylinositol (4, 5) diphosphate (PIP₂), leading to the formation of phosphatidylinositol (3, 4, 5) triphosphate (PIP₃) (Fig. 1C).

PIP₃ promotes the activation of the serine/threonine kinase AKT (PKB). AKT binds to PIP₃ through its pleckstrin homology domain (PHD) (Burgering et al. 1995;
Mutations in the components of this pathway: PTEN, AKT, PI3KCA are associated with tumorigenesis. Loss of PTEN, over-expression or constitutive activation of AKT and amplification of PI3KCA occur in several human cancers (Philp et al. 2001).

AKT (PKB) is a serine/threonine protein kinase involved in cell survival; therefore it plays an important role in tumorigenesis. Akt was originally identified as the oncogene in the transforming retrovirus AKT8 in the 1990’s. The AKT family consists of 3 isoforms: AKT1 (PKBα), AKT2 (PKBβ) and AKT3 (PKBγ), products of distinct genes located on chromosomes 14, 19 and 1 respectively. Their structure includes 3 domains: a NH2-terminal pleckstrin homology domain (PHD), a central kinase domain (KD) and a COOH-terminal regulatory domain containing the hydrophobic motif (HM) phosphorylation site (Fig. 2A). AKT1 is widely expressed. AKT2 is mostly expressed in insulin-target tissues such as liver, adipose tissue, muscle. AKT 3 is less expressed, being found mainly in brain and testes (Hanada et al, 2003; Coffer et al, 1998; Fayard et al, 2005).

AKT isoforms play an important role in various cellular responses, including cell survival/ apoptosis, cell cycle progression, transcription, motility and metabolic regulation. Most of the AKT substrates include the minimal consensus sequence RxRxx(S/T) where x is any amino acid and S/T is the phosphorylation site (Hanada et al, 2003; Fayard et al, 2005).
A. The PTEN protein (adapted from Leslie et al., 2004).

B. The PI3K: p85 – p110 heterodimer (adapted from Krasilnikov, 2000)

C. Molecular mechanism of action of the tumor suppressor PTEN as a direct antagonist of the PI3K/AKT pathway.
Phosphorylation of AKT is essential for its kinase function. AKT activity is induced in a PI3K-dependent manner. For AKT1, phosphorylation of both threonine 308 (in the kinase domain) and serine 473 (in the hydrophobic motif) are required for full kinase activation (Hanada et al., 2003; Coffer et al., 1998; Fayard et al., 2005).

AKT can be activated by growth factors, oncogenes, and cytokines (Burgering et al., 1995; Sizemore et al., 1999). Activated AKT phosphorylates and inactivates cell cycle inhibitory and pro-apoptotic substrates: GSK3, MDM2, BAD, pro-caspase-9, the family of forkhead (FH) transcription factors, protecting cells from apoptosis (Testa et al., 2001). Other substrates that control cell cycle promotion, angiogenesis, inhibition of apoptosis are also phosphorylated and activated by AKT including cyclins D1/D3, endothelial nitric oxide synthase (eNOS), human telomerase reverse transcriptase (hTERT), cellular inhibitors of apoptosis (cIAP) (Testa et al., 2001).

Also, it was shown that mice null for AKT1 show retarded growth and cells derived from these mice have increased susceptibility to several apoptotic stimuli (Chen et al., 2001). Knock-out of AKT2 in mice results in insulin resistance and severe diabetes, while knockout of AKT3 reduces brain size but does not affect growth or glucose metabolism.

The first identified physiological substrate of AKT was glycogen synthase kinase 3 (GSK3), which phosphorylates and inactivates glycogen synthase as a response to insulin stimulation. The two isoforms, GSK3α and GSK3β, are phosphorylated by AKT on serine residues in their NH₂-terminal region (serines 21 and 9 respectively) leading to their inactivation (Fig. 2B). Phosphorylation and consequently inactivation of GSK-3β
prevents degradation of cyclin D1 (required for progression of cells from the G1 into the S phase of the cell cycle). GSK3 is also involved in Wnt signaling (Hanada et al, 2003; Coffer et al, 1998; Fayard et al, 2005).

Bcl-2-associated death promoter (BAD) protein is a pro-apoptotic member of the Bcl-2 gene family which is involved in initiating apoptosis. Dephosphorylated BAD forms a complex with Bcl-2/ Bcl-xL localized on the mitochondrial membrane, inactivating them and thus allowing Bax/Bak-triggered apoptosis. AKT phosphorylates BAD on serine 136 (Fig. 2B), leading to the release of BAD and its sequestration in the cytosol by binding to the 14-3-3 protein, and therefore Bcl-2 is free to inhibit Bax-triggered apoptosis (Hanada et al, 2003; Coffer et al, 1998; Fayard et al, 2005).

AKT also induces transcription of genes that promote survival. AKT phosphorylates and activates IKK (Fig. 2B), leading to degradation of IκB, phosphorylation of NFκB subunits, and therefore the activated transcription factor NFκB is free to translocate in the nucleus and initiate transcription of prosurvival genes (Hanada et al, 2003; Fayard et al, 2005).

Murine double minute 2 (MDM2) is a negative regulator of the p53 tumor suppressor. Mdm2 is an E3 ubiquitin ligase that binds the p53 tumor suppressor and targets it to ubiquitination and degradation by proteasome and also acts as an inhibitor of p53 transcriptional activation. AKT-dependent phosphorylation of MDM2 (Fig. 2B) leads to nuclear localization of MDM2 leading to decrease p53 levels and p53-dependent transcription (Fayard et al, 2005).
Caspase 9 is an initiator and effector of apoptosis. AKT phosphorylates procaspase 9 on Ser 196 (Fig. 2B), therefore inhibiting the cytochrome C-induced cleavage of procaspase 9 and blocking the caspase cascade (Hanada et al, 2003; Fayard et al, 2005).

AKT phosphorylates the Forkhead (FH) family of transcription factors (FKHR, FoxO2, FKHR1 and AFX) on three highly conserved phosphorylation sites (Threonine 21, Serine 256 and Serine 318), leading to their exclusion from the nucleus and decreased transcriptional activity that is required to promote apoptosis (Fig. 2B). The target genes of the FH transcription factors include: Fas ligand, TRAIL (TNF related apoptosis inducing ligand) and TRADD (TNF receptor associated death domain) which promote apoptosis (Hanada et al, 2003; Fayard et al, 2005).

p21 is a cyclin-Cdk inhibitor that functions as a regulator of the cell cycle progression. Phosphorylation of p21 by AKT (Fig. 2B) inhibits nuclear localization of p21 and its ability to arrest the cell cycle (Hanada et al, 2003; Fayard et al, 2005).

p27 is another cyclin-Cdk inhibitor regulated by AKT-dependent phosphorylation (Fig. 2B). Phosphorylation of p27 results in exclusion of p27 from the nucleus and activation of cyclin-Cdk and cell cycle progression (Hanada et al, 2003; Fayard et al, 2005).

AKT phosphorylates the endothelial nitric oxide synthase (eNOS) on serine 1177 (Fig. 2B), leading to its enzymatic activation and production of nitric oxide (NO) which stimulates vasodilatation, vascular remodeling and angiogenesis (Hanada et al, 2003; Fayard et al, 2005).
A. Structure of the AKT (PKB) isoforms (adapted from Fayard et al. 2005).

B. Downstream substrates of the AKT pathway.

Fig. 2. A. Structure of the AKT (PKB) isoforms (adapted from Fayard et al. 2005).

B. Downstream substrates of the AKT pathway.
1.6. NFκB and regulation by the PI3K/AKT/IKK pathway

NFκB is a mainly anti-apoptotic transcription factor in cancer although various studies have provided evidence that NFκB may also function at times to promote apoptosis in certain cell types and conditions. NFκB was discovered as a lymphoid specific protein that binds to the oligonucleotide GGGACTTCC present in the enhancer of the Igκ (immunoglobulin K) light chain gene (Hayden et al, 2004; Verma et al, 1995; Bonizzi et al, 2004).

NFκB family consists of several protein members: p65 (RelA), Rel B, c-Rel, NFκB1 (p50/p105) and NFκB2 (p52/p100) (Fig. 3). The NFκB complex usually consists of two subunits which have promoter binding and transactivation properties. NFκB1 and NFκB2 proteins are synthesized as large precursors, p105 and respectively p100, which need to undergo processing by the ubiquitin/proteasome pathway in order to generate the mature p50 and p52 subunits (this involves selective degradation of their COOH-terminal region containing ankyrin repeats). NFκB proteins share an NH₂ – terminal conserved domain, about 300 amino acid long, called Rel homology domain, involved in dimerization, DNA binding, IκB binding (Fig. 3). The NFκB proteins are present in unstimulated cells as homo- or heterodimers bound to IκB inhibitors. The p65 and p50 subunits are ubiquitously expressed, while the RelB, c-Rel and p52 subunits play an important function in specific cell types (Hayden et al, 2004; Verma et al, 1995; Bonizzi et al, 2004; Thanos et al, 1995).
Fig. 3. **NFκB proteins** (adapted from Hayden & Ghosh, 2004). Rel HD = Rel Homology Domain, TA = Transactivation Domain, LZ = leucine zipper domain, GRR = glycine rich region.
Fig. 4. A. IκB proteins B. IkappaB Kinases (IKK) (adapted from Hayden & Ghosh, 2004). KD = kinase domain; LZ = leucine zipper domain; HLH = helix-loop-helix domain; NBD = NEMO-binding domain; α = α-helical domain; CC = coiled-coil domain; Z = zinc finger domain.
IκB family consists of seven members: IκBa, IκBβ, IκBe, IκBγ, BCL-3, p100 and p105, which share 5 - 7 conserved motifs called ankyrin repeats (30 - 33 amino acid long) and are required for interaction with NFκB proteins (Fig.3 & 4A) (Hayden et al, 2004; Verma et al, 1995; Bonizzi et al, 2004; Thanos et al, 1995).

NFκB family plays an important role in the inflammatory response (Hayden et al, 2004). In addition, NFκB also activates genes necessary for cellular survival, transformation, and oncogenesis. The typical NFκB complex consists of a p65-p50 heterodimer (Verma et al., 1995). p65, RelB, and c-Rel contain a transactivation domain (TA) located toward the C terminus so they have an intrinsic ability to drive transcription. p50 and p52 lack TAs but are involved in DNA binding (Liou et al., 1993). NFκB binding to its inhibitor, IκB, prevents NFκB from translocating to nucleus and the IκB - NFκB complex is sequestered as an inactive complex in the cytoplasm. Upon signaling, IκB is phosphorylated on two N-terminal serine residues and is degraded. This allows NFκB to freely translocate to the nucleus and bind to DNA (Bauerle et al. 1994; Siebenlist et al. 1994; Thanos et al. 1995; Verma et al. 1995) (Fig. 5). After entering the nucleus, NFκB binds to specific DNA sequences in the promoter or enhancer regions of NFκB-dependent target genes and interacts with co-activators and the basal transcriptional machinery to activate gene expression (Thanos et al. 1995; Verma et al. 1995). Various stimuli can induce NFκB including inflammatory cytokines, viral or bacterial products, and signals driven by activated RAS or other oncoproteins (Bonizzi et al, 2004; Verma et al. 1995; Hayden et al, 2004).
The IκB Kinase (IKK) complex is responsible for the phosphorylation of IκB, leading to its degradation by an ubiquitin-mediated process. The IKK complex contains two highly related serine/threonine kinase (catalytic) subunits, IKKα (IKK1) and IKKβ (IKK2), and a third structural (regulatory) subunit, IKKγ (NEMO) (Fig. 4B). In spite of the significant similarity between IKKα and IKKβ (over 50%), they do have some non-overlapping functions. IKKβ is the major catalytic subunit in the classical NFκB activation pathway by phosphorylating IκBα in response to cytokine stimulation (Bonizzi et al., 2004; Hayden et al., 2004).

The phosphorylation and degradation of IκB and the liberation of NFκB, however, are not sufficient to activate NFκB-dependent transcription (Martin et al., 2000; Sakurai et al., 1999; Sizemore et al., 2002; Sizemore et al., 1999; Wang et al., 1998; Wang et al., 2000). Our laboratory has shown that PI3K and AKT are part of another pathway, separate from IκB degradation, which is required for the phosphorylation and transactivation of p65/RelA NFκB by the IKK complex, leading to complete activation of NFκB (Sizemore et al., 2002; Sizemore et al., 1999).
Fig. 5. Activation of NFκB (P = phosphorylated; Ub = Ubiquitinated)
1.7. β-catenin and regulation by the PI3K/AKT/IKK pathway

Secreted glycoproteins of the Wnt family regulate many cellular processes, including cell fate decisions and cell proliferation. Activation of the Wnt signaling pathway results in the stabilization of β-catenin and consequent transcriptional activation of specific target genes. In normal tissues, β-catenin is involved in cell-cell adhesion through interaction with the cytoplasmic domain of E-cadherin (Giles et al. 2003; Polakis et al. 2000; Polakis et al. 2007). In the absence of Wnt signaling, the pool of non-adhesion-associated, soluble β-catenin forms a complex with the adenomatous polyposis coli (APC) tumor suppressor, axin and glycogen synthase kinase 3β (GSK3β). Phosphorylation of β-catenin by GSK3β targets it for ubiquitination and subsequent proteasomal degradation, thus keeping free cytosolic β-catenin levels low. Wnt signaling inactivates GSK3-β and consequently cytoplasmic β-catenin cannot be targeted for destruction, resulting in accumulation of β-catenin which is free to diffuse into the nucleus where it acts as co-activator for the T cell factor/lymphoid-enhancer factor (TCF/LEF) responsive genes (Fig. 6). This transcription factor complex plays a key role in the normal Wnt signaling pathway and its aberrant activation is associated with multiple human malignancies. Data from our lab has shown that IKKs also play a role in the phosphorylation and regulation of β-catenin. Data from IKK null MEFs, further supported by data from IKKα knockdown clones of the CRC cell line RKO, demonstrate a dramatic reduction in basal and Wnt dependent β-catenin dependent promoter activity (Behrens et al. 2004; Polakis et al. 2000; Polakis et al. 2007; Agarwal et al. 2005).
Fig. 6. Wnt Signalling pathway.
1.8. Role of the AKT/IKK/NFκB and β-catenin signaling pathways in ovarian cancer

One of the factors involved in deregulated growth, metastasis and resistance to apoptosis is the abnormal regulation of the AKT/IKK/NFκB pathway. Abnormal regulation of PI3K/AKT pathway contributes to cellular growth and survival. AKT is known to interact with cell cycle components like p21, p27, mTOR, GSK3. Previous studies have shown that AKT is over-expressed in invasive ovarian carcinomas and in ovarian cancer cell lines. AKT over-expression is associated with high FIGO stage and positive nodal status (AKT over-expression is associated with more advanced and aggressive cancer, poor prognosis, chemoresistance) (Noske et al. 2007). Use of LY294002, a PI3K-inhibitor, causes inhibition of phosphorylation of AKT and proliferation of ovarian cancer cells by inducing G1 cell cycle arrest as well as apoptosis (Gao et al. 2004; Shayesteh et al. 1999). Inhibition of PI3K by Wortmannin also increased the efficacy of Cisplatin in vivo. Treatment with a combination of Cisplatin and Wortmannin lead to a marked reduction in abdominal circumference, reduced production of ascites, inhibited tumor implantation in the liver and increased apoptosis in an athymic mouse ovarian cancer model (Ohta et al. 2006). Treatment with a combination of Paclitaxel and LY294002 (a second PI3K inhibitor) determined a marked inhibition of tumor growth, metastasis, and ascites production in athymic mice inoculated with a human ovarian cancer cell line (Hu et al. 2002). Additionally, inhibition of phosphorylation of Forkhead transcription factor (a substrate of AKT) sensitized human ovarian adenocarcinoma cells to Cisplatin (Arimoto-Ishida E et al. 2004). These studies
indicate that the PI3K/AKT pathway plays a role in ovarian cancer development, progression and chemotherapeutic resistance.

NFκB is known to regulate many genes that play important roles in cancer development and progression. NFκB is activated in several cancers and in response to chemotherapy and radiation. An NFκB inhibitor, BAY 11-7085, increased the ability of Paclitaxel and Cisplatin to inhibit tumor implantation into the liver and peritoneum, to inhibit cellular proliferation and cellular invasion (*Mabuchi et al. Nov 2004; Mabuchi et al. May 2004*). Also, paclitaxel activates IKKα activity in a human ovarian cancer cell line (*Huang et al. 2002*).

β-catenin plays a key role in the Wingless/Wnt signaling pathway and activation of this pathway is associated with multiple human malignancies including ovarian cancer. β-catenin mutations are common in endometrioid ovarian cancer (16-54%), but have also been found in mucinous ovarian cancer. These mutations are more frequent in low-grade, highly differentiated endometrioid ovarian cancers and are associated with a favorable prognosis and reduced capacity to metastasize. Mutations in the β-catenin gene (*CTNNB1*) that affect the N-terminal region of the protein (specific ser and thr residues and adjacent amino acids), essential for the degradation of β-catenin, have been reported in several ovarian cancer studies (*Catkus et al. 2004; Giles et al. 2003; Polakis et al. 2000; Sagae et al. 1999; Sanseverino et al. 2005; Willner et al. 2007; Wu et al. 2001*). Mutations at these sites would produce the same effect of increased free cytosolic β-catenin, leading to increased β-catenin-Tcf/Lef-dependent transcription. Mutations of APC and Axin have also been described in ovarian tumors (*Giles et al. 2003; Wu et al.*
Deregulation of this pathway leads to transcriptionally active $\beta$-catenin-Tcf/Lef complexes, which then activate a variety of target genes, including c-myc, cyclin D and MMP-7 (Zhai et al. 2002).

1.9. Cancer Chemotherapy

The beginnings of cancer chemotherapy go back to the early 20th century, when it was observed that during a military operation in WWII a group of people accidentally exposed to mustard gas were found to have very low white blood cell counts. The reasoning was that an agent that has a damaging effect on rapidly growing WBC might have a similar effect on cancer cells. In 1940’s, the first results were observed on patients with lymphoma that were given the drug iv.

Most chemotherapeutic drugs work by impairing cell division and by this mode of action they interfere with the fast – dividing cells. Some chemotherapeutics induce apoptosis. Combination chemotherapy of anticancer drugs is currently used to overcome the dosing limits of individual drugs and for their additive or synergistic cytotoxic effects. Chemotherapy is nowadays an important part in ovarian cancer treatment, in combination with surgery (B. G. Katzung, 2007; Brunton et al).

Ovarian cancer is a chemotherapy-sensitive disease and most patients diagnosed with this condition receive initial cytoreductive surgery followed by a chemotherapy regimen. During the last decades, several studies have tried to determine the regimens that are most effective in terms of drug activity and patient survival. Cisplatin was introduced in the treatment of ovarian cancer in 1970’s and has played since then an
important role in the treatment of this gynecological malignancy. Clinical trials have demonstrated that combination chemotherapy with cisplatin is superior to regimens without cisplatin. In 1990’s, combination regimens using paclitaxel and cisplatinum started to be used as standard therapy for ovarian cancer patients. Currently, several clinical trials are investigating the addition of a third drug to the paclitaxel- cisplatin standard treatment, and among these drugs are: topotecan, doxorubicin and gemcitabine. The next years should provide valuable data regarding other therapeutic agents that may be used in the treatment of ovarian cancer in order to achieve longer survival in patients and fewer relapses of the disease (Schorge et al, 2008; DeCherney et al, 2007).

1.9.1. Cisplatinum

Cisplatinum is a platinum-based, broad-activity chemotherapeutic drug used to treat various types of cancers: sarcomas, small cell lung cancer, ovarian cancer, bladder cancer, lymphomas and germ cell tumors (Brunton et al). Cisplatinum is considered a non-cell cycle specific alkylating-like agent. Cisplatin leads to cell cycle arrest in G2 and induces apoptosis or programmed cell death

The compound cis-PtCl₂(NH₃)₂ was initially described as “Peyrone's salt” by M. Peyrone in 1845. Alfred Werner described cisplatinum’s structure in 1893 (Fig. 7A); in 1960s Barnett Rosenberg and van Camp observed that it inhibited the binary fission in E. coli and in 1978 cisplatinum was approved by FDA for clinical use.

Cisplatin enters the cells by passive diffusion and its two chloride groups are replaced by water, forming the active species (positively charged) which react to DNA,
leading to formation of intrastrand (over 90%) and interstrand crosslinks, interfering with DNA replication and cell division. The damaged DNA elicits DNA repair mechanisms, which in turn activate apoptosis when repair proves impossible (Cisplatin SIGMA Product information; B. G. Katzung, 2007; Brunton et al).

Cisplatin is often used as a part of combination chemotherapy and it has, like all other drugs, dose dependent and dose limiting side effects, such as: nephrotoxicity, neurotoxicity (demyelination, distal sensory neuropathy), ototoxicity (hearing loss, tinnitus) and gastrointestinal toxicity (nausea, vomiting, anorexia) (Cisplatin SIGMA Product information; B. G. Katzung, 2007; Brunton et al).

1.9.2. Paclitaxel

Paclitaxel (Taxol) was discovered in a NCI program in 1967 by M.E. Wall and M.C. Wani who isolated it from the bark of the Pacific yew tree (Taxus brevifolia). Currently, all paclitaxel production is synthetic, using plant cell fermentation technology. Paclitaxel is used for the treatment of patients with lung, ovarian, breast, prostate, head and neck cancer and Kaposi sarcoma (Rowinski et al, 1995).

Paclitaxel (fig. 7B) is an alkaloid ester used for the treatment of cancer and it acts as a mitotic inhibitor by binding with high affinity to microtubules, promoting tubulin polymerization and stabilizing microtubules to depolymerization. Microtubules are involved in several eukaryotic cell functions, such as maintaining the cell shape, cell mobility, attachment, intracellular transport, mitosis (mitotic spindle).
Fig. 7. A. Chemical structure of Cisplatinum.

B. Chemical structure of Paclitaxel (adapted from Frense D., 2007).
By altering the cellular equilibrium between the tubulin dimers and microtubule formation and degradation, paclitaxel affects the cell division and all cellular processes dependent on microtubules (Rowinski et al, 1995; Brunton et al).

Paclitaxel is a cell-cycle specific drug and it induces cell cycle arrest during G2 and M phases (which are the most sensitive to radiation). It also inhibits the transition from G0 to S phase. (Paclitaxel SIGMA Product information; B. G. Katzung, 2007; Brunton et al).

Paclitaxel is insoluble in aqueous solution and over 95% is bound to plasma protein; it is metabolized in the liver by cytP450, therefore a limiting condition in administration of this drug is hepatic dysfunction. Side effects include nausea vomiting, loss of appetite, joint pain, muscle aches, hair loss, bruising or bleeding at the injection site (Paclitaxel SIGMA Product information; B. G. Katzung, 2007; Brunton et al).

1.10. Specific aims of this dissertation

One of the first steps involved in the development of a malignancy is the acquired capacity of a cell to escape death. Cells die through an orderly process called apoptosis or programmed cell death, process by which a cell commits “suicide” through destruction of their DNA and other cellular components. Malignant cells are able to overcome apoptotic pathways by up-regulating gene products that inactivate the cell death machinery. The identification of the molecular mechanism of action of the tumor suppressor gene PTEN has revealed the important role that the phosphatidylinositol 3’ kinase (PI3K)/AKT pathway plays in tumorigenesis. Our laboratory has delineated a direct role of the
PI3K/AKT and PTEN pathways in regulation of the anti-apoptotic transcription factor nuclear factor kappa B (NFκB). We have shown that AKT and PTEN mediate regulation of NFκB by controlling the ability of the IκB kinases (IKKs) to phosphorylate and activate the p65 subunit of NFκB. Loss of normal regulation of NFκB and the IκB kinases (IKKs), which control the activation of NFκB, is becoming more apparent as a major pathway promoting tumorigenesis. We have additional data that the IKKs also phosphorylate and control the function of the transcriptional regulator β-catenin, another important contributing factor in the development of cancer. The following hypothesis links these signaling and transcriptional factor pathways, separately known to play important roles in ovarian cancer, together.

The experiments in this dissertation are designed to test the hypothesis that inappropriate activation of the PI3K/AKT/IKK pathway in ovarian cancer alters the transcriptional activation of both NFκB and β-catenin and that the consequence of these alterations in transcription are important in tumorigenesis, facilitating cellular transformation and resistance to apoptosis. Therefore, interruption of the AKT/IKK pathway should inhibit the tumorigenic, invasive and metastatic capacity as well as alter the chemotherapeutic resistance of ovarian cancer cells (fig. 8).

The objectives of this hypothesis will be accomplished through the following specific aims:

**Specific Aim 1:** To examine the role of AKT1 and IKKβ in the regulation of the NFκB and β-catenin signal transduction pathways in ovarian cancer cells.
Specific Aim 2: To examine the role of AKT1 and IKKβ in ovarian cancer cell proliferation, anchorage-independent growth, migration, and invasion.

Specific Aim 3: To assess the roles of AKT1 and IKKβ in ovarian cancer chemotherapeutic resistance.
MODEL SYSTEM
Genes Down-Regulated by Inhibition of AKT/IKK/NFκB and β-catenin Pathway in Cancer Cell Lines

Fig. 8. Model system for ovarian cancer cell lines with inappropriate constitutive activation of AKT and IKKβ. The ovarian cancer cell lines are engineered to overexpress either RNAi to AKT or IKKβ to block constitutive AKT or IKKβ activity and subjected to DNA microarray analysis to screen for genes dependent on the AKT/IKK/NFκB and β-catenin pathway. Also, studies of the signaling pathways, apoptosis and cell cycle arrest in response to chemotherapeutics (in-vitro), tumorigenesis and invasiveness of the engineered cancer cells are proposed in this model.
CHAPTER 2

MATERIALS AND METHODS

Cell lines and reagents.

The cell lines, SKOV-3, A2780, Hey, OVCA429, L-cells, Wnt3a-secreting L-cells (L-Wnt3A cells), MCF-7, and MDA-MB-231 cells were purchased from ATCC (Manassas, VA). L-Wnt3a (ATCC #CRL-2647) and L-cells (ATCC #CRL-2648) were grown in DMEM medium and conditioned medium was generated according to the depositor’s instructions. Conditioned medium from L-Wnt3a- cells was harvested, filtered, and used for stimulation of cells with Wnt3a. Filtered conditioned medium from L-cells was used as a control. All other cells were maintained in RPMI 1640 (1X) medium, supplemented with 10% fetal bovine serum (FBS), 100 µg/ml penicillin G, and 100 µg/ml streptomycin. The AKT1 and IKKβ shRNA expressing knock-down cell lines were obtained from the parental lines A2780 and SKOV-3. A2780 AKT1 p1, A2780 AKT1 c2, A2780 AKT1 c13, A2780 IKKβ p1, A2780 IKKβ c3, A2780 IKKβ c8 were maintained in the RPMI 1640 medium detailed above with 400µg/ml G418. SKOV-3 AKT1 p1 was cultured in the RPMI 1640 medium detailed above with 100µg/ml G418. SKOV-3 AKT1 c7, SKOV-3 AKT1 c10, SKOV-3 IKKβ p1, SKOV-3 IKKβ c8, SKOV-3 IKKβ c13, SKOV-3 IKKβ c14 were maintained in the RPMI 1640 medium detailed above with 200µg/ml G418. All cell cultures were maintained at 37° C in a humidified atmosphere with 10% CO₂.
RPMI 1640 (1X) (with L-glutamine), RPMI 1640 (2X), DMEM (1X) (4.5g/L glucose, with L-glutamine) media, trypsin – EDTA (1X) (0.05% trypsin, 0.53 mM EDTA·4Na in HBSS w/oCa++, w/o Mg++, Penicillin - Streptomycin (5000 units/ ml penicillin G, 5000 μg/ ml streptomycin sulfate in 0.85 % NaCl) were purchased from the Media Lab, Central Cell Services, LRI, CCF.

Fetal Bovine Serum (FBS) was purchased from SERUM SOURCE INTERNATIONAL, INC. (Charlotte, NC). Lipofectamine 2000 was purchased from INVITROGEN CO (Carlsbad, CA). G418 sulfate was purchased from MEDIATECH, INC. TNFα was obtained from PeproTech (Rocky Hill, NJ). Paclitaxel and Cisplatinum were purchased from SIGMA (St Louis, MO). CellTiter 96® AQueous One Solution Cell Proliferation Assay and Dual-Luciferase® Reporter Assay System were purchased from PROMEGA CO (Madison WI). Guava ViaCount Reagent, Guava Cell Cycle Reagent and Guava TUNEL Kit were purchased from GUAVA TECHNOLOGIES, INC. DMSO was obtained from Sigma (St. Louis, MO).

Antibody to β−catenin was from BD Bioscience (San Jose, CA). The β-actin antibody was from Sigma (St. Louis, MO). Antibodies to p65 nuclear factor kappa B (NFκB), p50 nuclear factor kappa B (NFκB), IκBα and β ig-h3 were from SANTA CRUZ BIOTECHNOLOGY, INC. (Santa Cruz, CA). Antibodies to AKT1, phosphorylated AKT (serine 473), phosphorylated IκBα (serines 32 and 36), IKKα and IKKβ were from Cell Signaling (Danvers, MA). Antibody to SPARC was from ZYMED Laboratories (San Francisco, CA).
**Short interfering RNA.**

To perform stable RNAi knockdown of AKT and IKKβ in our ovarian cancer cell lines, we utilized the mammalian expression vector, pSUPER from OligoEngine (Seattle, WA) for stable expression of short hairpin RNA (shRNA) for AKT1 and IKKβ. RNAi technology is an important tool for both *in vitro* and *in vivo* use in mammalian genetics and for new gene therapy approaches.

Figure 9 illustrates a simplified mechanism of RNA interference.

For knockdown of AKT and IKKβ in our ovarian cancer cell lines, we utilized the mammalian expression vector, pSUPER from OligoEngine (Seattle, WA) that allows for stable expression of short hairpin RNA (shRNA). The gene-specific inserts include the 19-nucleotide sequence corresponding to nucleotides 1943-1961 downstream of the transcription start site (gctgctgggcaaaggcact) of AKT1 or nucleotides 1488-1506 downstream of the transcription start site (gtacagcgagcaaaccgag) of IKKβ, that are separated by a 9-nucleotide non-complementary spacer (tctcttgaa) from the reverse complement of the same 19-nucleotide sequence. These vectors were referred to as pSUPER-AKT and pSUPER-IKKβ respectively. These sequences were inserted into the pSUPER backbone after digestion with BglII and HindIII and transformed into BL21-A1 One Shot™ supercompetent cells from Invitrogen according to the manufacturer's instructions. Several clones were obtained, and the vectors were amplified and sequence was verified.
**Fig. 9. Simplified mechanism of RNA interference** (adapted from OligoEngine website). RNAi is a process by which gene expression can be silenced through the addition of double stranded RNA (dsRNA). dsRNA enters the cell and it is cleaved by an RNase III–like enzyme (Dicer) into double stranded small interfering RNAs (siRNA). These siRNAs are 21-23 nucleotides long and contain 2 nucleotide overhangs on the 3’ end. siRNAs integrate into a multi-subunit protein complex known as the RNAi induced silencing complex (RISC), whose function is to guides the siRNAs to the target RNA sequence. The siRNA duplex unwinds and the antisense strand remains bound to RISC and directs degradation of the complementary mRNA sequence by a combination of endo- and exo-nucleases.
**Western Blot Analysis**

Cells, untreated or treated with TNFα, L-cells or Wnt3a conditioned media or chemicals, were washed three times with phosphate-buffered saline (PBS) and lysed with lysis buffer including 1% Triton X-100. Equal amounts of cell extracts were fractionated by SDS–PAGE and transferred to the Immobilon-P membranes (Millipore, Bedford, MA). Analysis was performed with the indicated primary antibodies and visualized with horseradish peroxidase-coupled goat anti-rabbit or anti-mouse immunoglobulins, using the ECL Western blotting detection system from PerkinElmer Life Sciences (Boston, MA).

**Electrophoretic Mobility Shift Assay (EMSA).**

The DNA binding capacity of NFκB was measured by EMSA. For the EMSA analysis, nuclear extracts were prepared at 0 and 30 minutes after treatment with 20 ng/ml TNF-α for NFκB. The NFκB binding site from the immunoglobulin gene promoter was synthesized by ITD Technology Inc. (Coralville, IA) and used as probes. EMSA and the supershift assays were performed on nuclear lysates as previously described (Sizemore et al., 2002). The DNA complexes were separated on 5% polyacrylamide gels by electrophoresis and then visualized by autoradiography.

**Transfection and promoter assays.**

β-catenin/TCF/LEF- or NFκB-dependent transcriptional activity was determined as described previously (Agarwal et al., 2005). Exponentially growing cells in 24-well
plates were transfected with either the β-catenin/TCF/LEF- (pOT) or NFκB-dependent (NFκB) luciferase reporter construct with Lipofectamin 2000 (Invitrogen, Carlsbad, California). Luciferase reporter plasmids for pOT and NFκB were gifts from Dr. Bert Vogelstein (John Hopkins, Baltimore, MD) and Dr. Bryan Williams (Monash Institute of Medical Research, Clayton Victoria, Australia) respectively. The β-catenin/ TCF4 reporter construct pGL3-OT (here referred to as pOT) consists of 3 TCF binding sites upstream a basic promoter (TATA box) that drives firefly luciferase expression. The NFκB-dependent reporter plasmid p5XIP10κB contains 5 tandem copies of the NFκB site from the IP10 gene. The renilla luciferase construct pRL-TK (Promega, Madison, WI) was co-transfected as a normalization control. After an overnight incubation, the cells were stimulated with either control, TNFα, or Wnt3a medium, harvested, and lysed. For β-catenin-dependent promoter activity, cells were treated with either L- or Wnt3a-medium for 24 hrs. For NFκB-dependent promoter activity, the cells were then treated with or without 20 ng/mL of TNFα for 4-6 h. 20 μl of each lysate was incubated with luciferase substrates for firefly and Renilla and luciferase assays were performed with the dual luciferase reporter assay system (Promega). The β-catenin/TCF/LEF- or NFκB-dependent firefly luciferase activity was normalized with the value of the corresponding renilla-dependent luciferase activity and the ratio (n = 3, mean ± S.D.) was statistically analyzed.
**Soft Agar Assays (to measure anchorage independent growth)**

We suspended 10,000 cells in 1.2% agarose in RPMI 1640 2X containing 20% serum and 2X P/S and plated them into 6 cm dishes on 2.4% agarose in the same medium. The next day after plating, 2mls of RPMI 1640 (1X) were added to each plate, and the media was changed twice a week. One week after plating (for A2780 parent and knockdown lines) and respectively two weeks after plating the cells (for SKOV-3 parent and knockdown lines), the plates were analyzed for soft agar anchorage-independent growth. Colonies were stained with Crystal Violet, pictures were taken and colonies were counted. Two dishes per cell line were used for each experiment and the experiment was repeated at least three times.

**Migration and Invasion assays.**

To investigate their migratory and invasive potential, cells were plated either in migration chambers (BD BioCoat Control Inserts) or in matri-gel invasion chambers (BD BioCoat Matrigel Invasion Chambers) of 24-well format (BD Bioscience, San Jose, CA), at 1 x 10^5 cells (A2780 parent and knockdown lines) or respectively at 1 x 10^4 cells (SKOV-3 parent and knockdown lines). The migratory and invasive capacities of cell lines were assessed after 24 hours for the A2780 cells or after 6 hours (migration) or 24 hours (invasion) for the SKOV-3 cells. The migrating and invading cells were fixed with methanol and stained with Harris Modified Hematoxylin (Fisher Scientific). Images were taken at the Imaging Core, Lerner Research Institute, CCF. The number of migrating and
invading cells were counted and statistically analyzed (n = 3, mean ± S.D.). All tests were repeated at least twice with n = 3 in each experiment.

**MTS assay – for cell proliferation.**

The cells were plated at 5000/ well (for A2780 parent and knockdown clones) or 1000/ well (for SKOV-3 parent and knockdown clones) on 96-well plates. The next day the cells were treated with either plain media and TNF-α, or L cells media and Wnt3a media. The proliferation of the cells was evaluated after 24, 48 and 72 hours by using the CellTiter 96® AQ™ueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer’s protocol. The CellTiter 96® AQ™ueous One Solution Cell Proliferation Assay is a colorimetric method for determining the number of viable cells in proliferation or cytotoxicity assays. It contains a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine ethosulfate; PES) which combines with MTS to form a stable solution. The MTS tetrazolium compound is bioreduced by metabolically active cells into a colored formazan product which is soluble in tissue culture medium and the absorbance at 490nm is recorded with a 96-well plate reader. The quantity of formazan formed is directly proportional to the number of living cells in culture.

**MTS assays - to measure viability after drug treatment**

The cells were plated at 5000/ well (for A2780 parent and knockdown clones) or 1000/ well (for SKOV-3 parent and knockdown clones) on 96-well plates. The next day
the cells were treated with either plain media (as control) or various drug concentrations. The effects of the drugs were evaluated after 48 hours using the CellTiter 96® AQeous One Solution Cell Proliferation Assay (Promega) according to the manufacturer’s protocol.

**Cell proliferation assay using Guava ViaCount Assay.**

Equal numbers of cells were plated in 10-cm culture plates. After 24, 48 and 72 hrs, the cells and media were collected according to the Guava ViaCount Reagent recommended protocol, stained and analyzed on the Guava PCA System. The experiment was done in triplicates.

**Cell cycle analysis using Guava Cell Cycle Analysis**

Cells of the A2780 parent and knockdown pools were plated in 10-cm culture plates for 24 hrs. Next, the cells were synchronized in G0 by culturing in serum free media for 36 hrs. After synchronization, the plates were treated with either RPMI 1640 1X (serum containing) media (control) or with RPMI 1640 1X (serum containing) + PCTX 30 nM for 24 hrs. After 24 hrs, the cells and media were collected according to the Guava Cell Cycle Reagent recommended protocol, fixed, stained and analyzed on the Guava PCA machine to determine the percentage of cells in specific stages of the cell cycle based on the DNA content and sub-diploid fraction of cells in each line. The experiment was done in triplicates.
**Apoptotic Assay using Guava ViaCount Reagent**

Equal numbers of cells were plated in 10-cm culture plates. After 24 hrs, the plates were either left untreated (as control) or treated with PCTX 30 nM (for A2780 parent and knockdown pools) or with CPT 25 μM (for SKOV-3 parent and knockdown pools). After 48 hrs, the cells were collected and counted using the Guava ViaCount Reagent (Guava Technologies) on a Guava PCA machine according to manufacturer’s protocol to determine the percentage of cells that are apoptotic and necrotic. The experiment was done in triplicates. The ViaCount Assay distinguishes viable and non-viable cells based on differential permeabilities of two DNA-binding dyes contained by the reagent. The nuclear dye stains only nucleated cells, while the viability dye brightly stains dying cells.

**Apoptotic Assay using Guava TUNEL Kit**

Equal numbers of cells were plated in 10-cm culture plates. After 24 hrs, the plates were either left untreated (as control) or treated with PCTX 30 nM (for A2780 parent and knockdown pools) or with CPT 25 μM (for SKOV-3 parent and knockdown pools). After 48 hrs, the cells were collected and analyzed using the Guava TUNEL Kit (Guava Technologies) on a Guava PCA machine according to manufacturer’s protocol to determine the percentage of cells that are apoptotic and necrotic. The experiment was done in triplicates.
Statistical Data Analysis

Mean values between groups will be compared using a repeated measures ANOVA to account for the triplicate experiments. Statistical significance of differences will be evaluated by one-way ANOVA with Bonferroni correction or Fisher's exact probability test. Values were considered significantly different when $p < 0.05$. 
CHAPTER 3

RESULTS

Specific Aim 1: To examine the role of AKT1 and IKKβ in the regulation of the NFκB and β-catenin signal transduction pathways in ovarian cancer cells.

1. Pathway screening in the ovarian cancer cell lines

In order to examine the regulation of the NFκB and β-catenin transcription pathways by AKT and IKKβ, we needed to first find the appropriate ovarian cancer cell lines in which to set up our model system to inhibit these two pathways. Our criteria for selection was that the ovarian cancer cell lines must have evidence of either constitutive or inducible activation of AKT and/or IKK. We screened two breast cancer cell lines (MDA MB 231 and MCF-7) and four ovarian cancer cell lines (SKOV-3, Hey, A2780, OVCA429) for activation of these pathways. Western blot analysis (Fig.10) was used to quantify the level of IKKa, IKKβ, phosphorylated AKT (serine 473), AKT1, phosphorylated IkBα (serines 32 and 36), IkBα, p65 NFκB, β-catenin and β-actin (as loading control). Our screening of the cell lines showed that both the SKOV-3 and A2780 ovarian cell lines have activated AKT, which fit our first criteria. Although, the levels of p65 NFκB did not vary between the ovarian cancer cell lines there were clues that perhaps the NFκB pathway was activated as indicated by the mobility shift of IkBα...
(the appearance of two bands instead of one) and reaction with the phospho-specific antibody for IκBα which is indicative of phosphorylation by the IKKs. Interestingly, the screening showed that the SKOV-3 cells have activated AKT, lack IKKα, have indications of activated NFκB and β-catenin while the A2780 cells have activated AKT, have indications of activated NFκB, but express a very low basal level of stabilized β-catenin. Therefore, we further investigated the ovarian cancer cell lines for constitutive and inducible NFκB and β-catenin transcriptional activation.
Fig. 10: Pathway screening of breast cancer cell lines (MDA MB 231, MCF7) and ovarian cancer cell lines (SKOV3, Hey, A2780, OVCA429). Cells were lysed and analyzed by Western Blot for levels of pAKT, total AKT, IKKα, IKKβ, pIkBα, IkBα, p65 NFκB and β-catenin using the respective primary antibodies. β-actin was used as loading control. pAKT = phospho-AKT, pIkBα = phospho-IκBα.
To measure NFκB transcriptional activation, we performed two assays. One assay is to measure the activated nuclear NFκB complexes present in the nucleus of the ovarian cancer cell lines by electrophoretic mobility shift analysis (EMSA) and the other is to measure NFκB-dependent promoter activity using a promoter luciferase reporter assay. First, to measure nuclear activated NFκB complexes, we performed NFκB Electrophoretic Mobility Shift Analysis (EMSA) on nuclear extracts of the four ovarian cancer cell lines (Fig. 11A). Cytoplasmic and nuclear extracts were prepared at time 0 (C) and 30 min (T) after treatment with 20 ng/ml TNF-α. Also to confirm the composition of the NFκB complexes, an antibody supershift of the NFκB EMSA complexes was performed in which an NFκB subunit specific antibody (either to p65 or p50 NFκB) is added to the protein-DNA binding reaction that causes slowing of the NFκB-DNA complex in the gel if that NFκB subunit is present in the complex. This data indicates that all the ovarian cancer cell lines have basal and TNF-α-inducible NFκB DNA binding activities.

Next NFκB-dependent promoter activity in the ovarian cancer cell lines was assessed by promoter reporter assay. Briefly, cells were transfected with the NFκB-dependent reporter construct and 24 hours later cells were treated with (TNF) and without (C) 20ng/ml TNFα, lysed and assayed 4 hours later (Fig.11). SKOV-3 cells have a higher basal level of NFκB transcriptional activity slightly induced by TNFα, while A2780 cells have a lower basal level of NFκB transcriptional activity that is significantly induced by TNF-α treatment (Fig. 11B). This data indicated that both the SKOV-3 and A2780 cell
lines demonstrate inappropriate basal activation of NFκB DNA binding and NFκB-dependent promoter activity.

To measure β-catenin transcriptional activation, we performed only the β-catenin-dependent promoter activity using a promoter luciferase reporter assay, as the EMSA for β-catenin was not fully successful on these cell lines’ assays. Briefly cells were transfected with the β-catenin-dependent reporter construct and 24 hours later cells were treated with Wnt-3a (W) or control (L) medium, lysed and assayed 24 hours later. SKOV-3 cells have a medium level of β-catenin transcriptional activity that was not inducible by Wnt-3a, while A2780 cells have a lower basal level of β-catenin transcriptional activity that is significantly induced by Wnt-3a (Fig. 11C). Interestingly, this data showed that the SKOV-3 and A2780 ovarian cancer cells have both basal and inducible NFκB and β-catenin transcriptional activities in addition to activated AKT. Therefore, we chose these two ovarian cancer cell lines to set up our cell model systems to investigate the loss of AKT1 and IKKβ on the regulation of NFκB and β-catenin signal transduction pathways, the tumorigenic properties, and chemotherapeutic resistance of ovarian cancer cells.
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Fig. 11. NFκB and β-catenin transcriptional activation in ovarian cancer cell lines.

A. NFκB Electrophoretic Mobility Shift Analysis (EMSA) of 4 ovarian cancer cell lines (OVCA429, SKOV-3, Hey, A2780) showing TNF-α inducible NFκB binding activities for all cell lines. Nuclear extracts were prepared at time 0 (control samples, C) and 30 min after treatment with 20 ng/ml TNF-α (treated samples, T). For the A2780, SKOV-3, and Hey ovarian cancer cell lines, supershifts with antibodies to p50 NFκB (T+p50) or p65 NFκB (T+p65) antibodies were performed as indicated. B. NFκB-dependent promoter activity in ovarian cancer cell lines. Cells were transiently co-transfected with a NFκB-dependent luciferase reporter construct and then stimulated with TNF-α. Cells were harvested 4-6 hrs later and luciferase activity was determined. C = control (untreated samples). TNF = TNF-α treated samples. Data are expressed as mean normalized luciferase promoter activity ± SD, n=3. C. β-catenin-dependent promoter activity in ovarian cancer cell lines. Cells were transiently co-transfected with pOT luciferase reporter construct and then stimulated with Wnt3a. Cells were harvested 24 hrs later and luciferase activity was determined. L = control samples (treated with L-cells conditioned media). W = Wnt3a treated samples. Data are expressed as mean normalized luciferase activity ± SD, n=3
2. Stable shRNA knock down of AKT1 and IKKβ in SKOV3 and A2780 ovarian cancer cell lines

To set up our cell model system to examine the loss of AKT1 and IKKβ on ovarian cancer, we chose to utilize a stable form of RNAi using a shRNA vector. The pSUPER-AKT and pSUPER-IKKβ shRNA plasmids were stably transfected into the A2780 and SKOV3 cell lines and several G418-resistant clones and pools were selected for cells whose cellular expression of IKKβ and AKT1 has been significantly and stably knocked down. The specific shRNA knock-down cell lines that have been established will be used to investigate specific effects of loss of either IKKβ or AKT1 on the various tumorigenic pathways and phenotypes of the ovarian cancer cell lines. To verify the presence and specificity of the shRNA knockdown, a sample from each cell line was lysed and total cellular protein was extracted. Western blots were used to screen for potential AKT1 and IKKβ shRNA expressing SKOV3 and A2780 cell pools and clones (Fig 12A, B). This screening yielded several clones and a pool of clones from each ovarian cancer cell line with specific and efficient knockdown of either AKT1 and IKKβ. The levels of expression in the stable pools and clones were much lower or undetectable as compared to the parental control ovarian cancer cell lines. We found that the shRNA knockdown clones and pools for AKT1 and IKKβ serve as good controls for each other as they specifically only target AKT1 and IKKβ respectively and don't affect the levels of the other non-targeted protein or of IKKα. We used these pools and clones of SKOV-3 and A2780 ovarian cancer cells with significantly reduced expression of either AKT1 or IKKβ for our further studies.
Fig. 12. Screening for AKT1 and IKKβ shRNA expressing SKOV-3 ovarian cancer cell line pools and clones. Cells were lysed and analyzed by Western Blot to quantify the expression of phosphorylated AKT1 (pAKT), AKT1, IKKα and IKKβ using the respective primary antibodies to screen for potential AKT1 and IKKβ shRNA expressing cell pools and clones for the SKOV-3 (A) and A2780 (B) ovarian cancer cell lines. SKOV3 = parental control cell line, SKOV-3 AKT1 p1 = SKOV-3 AKT1 knockdown pool 1, SKOV-3 AKT1 c7 = SKOV-3 AKT1 knockdown clone 7, SKOV-3 AKT1 c10 = SKOV-3 AKT1 knockdown clone 10 SKOV-3 IKKB1p1 = SKOV-3 IKKβ knockdown pool 1, SKOV-3 IKKB1c8 = SKOV-3 IKKβ knockdown clone 8, SKOV-3 IKKB1c13 = SKOV-3 IKKβ knockdown clone 13, SKOV-3 IKKB1c14 = SKOV-3 IKKβ knockdown clone 14, A2780 = parental control cell line, A2780 AKT1p1 = A2780 AKT1 knockdown pool 1, A2780 AKT1c2 = A2780 AKT1 knockdown clone 2, A2780 AKT1c13 = A2780 AKT1 knockdown clone 13, A2780 IKKB1p1 = A2780 IKKβ knockdown pool 1, A2780 IKKB1c3 = A2780 IKKβ knockdown clone 3, A2780 IKKB1c8 = A2780 IKKβ knockdown clone 8.
3. Loss of IKKβ or AKT1 significantly inhibits both the inappropriate basal and TNFα-induced NFκB transcriptional activity in SKOV-3 ovarian cancer cell line

We next examined the regulation of the NFκB transcription pathway by AKT and IKKβ utilizing NFκB EMSA and NFκB-dependent promoter analysis in the AKT1 and IKKβ shRNA expressing SKOV3 pools and clones. First, we performed NFκB EMSA on nuclear extracts of the SKOV-3 parental and AKT-1 and IKKβ knockdown cell line pools and clones (Fig. 13A). Nuclear extracts were prepared at time 0 (C) and 30 min (T) after treatment with TNF-α and NFκB DNA binding was measured. These data show that loss of either IKKβ or to a lesser extent AKT1 reduces TNF-α-induced NFκB DNA binding.

Next, NFκB-dependent promoter activity in the SKOV-3 parental and knockdown lines was assessed by promoter reporter assay. All the AKT-1 and IKKβ-knockdown SKOV-3 pools and clones have significantly lower basal (C) and TNF-α (T) induced NFκB promoter activity compared to the parental line (Fig. 13B). This data indicates that loss of IKKβ or AKT1 significantly inhibits both the inappropriate basal and TNFα-induced NFκB transcriptional activity in the SKOV-3 ovarian cancer cell line.
A.

B.

Normalized Luciferase Activity

![Graph showing normalized luciferase activity for different conditions and cell lines.](image-url)
Fig. 13. The effect of AKT1 or IKKβ loss on the NFκB transcriptional activation in the SKOV-3 ovarian cancer cell line. A. NFκB Electrophoretic Mobility Shift Analysis (EMSA) of SKOV-3 ovarian cancer parental control cell line and AKT1- and IKKβ-knockdown pools and clones. Nuclear extracts were prepared at time 0 (control samples, C) and 30 minutes after treatment with TNF-α (treated samples, T). B. NFκB-dependent promoter activity in SKOV-3 ovarian cancer parental control cell line and AKT1- and IKKβ-knockdown pools and clones. Cells were transiently co-transfected with a NFκB-dependent promoter reporter construct. After 24 hours, the cells were either unstimulated (C) or stimulated with TNF-α (T). The cells were harvested 4-6 hrs later and luciferase activity was determined. Data is expressed as mean normalized luciferase activity ± SD, n=3. SKOV3 = parental control cell line, SKOV-3 AKT1 p1 = SKOV-3AKT1 knockdown pool1, SKOV-3 AKT1 c7 = SKOV-3 AKT1 knockdown clone 7, SKOV-3 AKT1 c10 = SKOV-3 AKT1 knockdown clone 10, SKOV-3 IKKB1p1 = SKOV-3 IKKβ knockdown pool 1, SKOV-3 IKKB1c8 = SKOV-3 IKKβ knockdown clone 8, SKOV-3 IKKB1c13 = SKOV-3 IKKβ knockdown clone 13, SKOV-3 IKKB1c14 = SKOV-3 IKKβ knockdown clone 14
4. Loss of IKKβ or AKT1 significantly inhibits the TNFα-induced NFκB transcriptional activity and the loss of IKKβ significantly inhibits both the basal and Wnt-stimulated β-catenin-dependent transcriptional activity in A2780 ovarian cancer cell line

We next examined the regulation of the NFκB transcription pathway by AKT and IKKβ utilizing NFκB EMSA and NFκB-dependent promoter analysis in the AKT1 and IKKβ shRNA expressing A2780 pools and clones. First, we performed NFκB EMSA on nuclear extracts of the A2780 parental and AKT-1 and IKKβ knockdown cell lines pools and clones (Fig. 14A). Nuclear extracts were prepared at time 0 (C) and 30 min (T) treatment with TNF-α and NFκB DNA binding was measured. TNF-α -induced NFκB DNA binding is reduced in only in the IKKβ knockdown cell lines compared to the parental line whereas the AKT1 knockdown cell lines had equivalent levels as the parent line.

Next, NFκB-dependent promoter activity in the A2780 parental and knockdown lines was also assessed by promoter reporter assay. All the AKT1 and IKKβ knockdown clones have lower TNF-α-induced (T) promoter activity compared to the parental line with the effect of the IKKβ knockdown being more severe (Fig. 14B). This data indicates that loss of either IKKβ or AKT1 lowers the TNFα-induced NFκB transcriptional activity. However, the loss of either AKT1 or IKKβ in A2780 cells does not reduce NFκB activity as significantly as it does in the SKOV3 cells. This may indicate that perhaps IKKα plays an important role in NFκB activation in the A2780 ovarian cancer cell line.
Next, to measure β-catenin transcriptional activation of the A2780 parental and knockdown lines, we performed β-catenin-dependent promoter activity using a promoter luciferase reporter assay. Briefly, cells were transfected with the β-catenin-dependent reporter construct and 24 hours later cells were treated with Wnt-3a (W) or control (L) medium, lysed and assayed 24 hours later. All the IKKβ–knockdown cells have significantly lower basal and Wnt3a-induced activities compared to the parental line (Fig. 14C), while AKT1 knockdown had no significant effect. This data indicates that loss of IKKβ significantly inhibits both the basal and Wnt-stimulated β-catenin-dependent transcriptional activity.

The data obtained for specific aim #1 indicate that loss of the either AKT1 or IKKβ inhibits the inappropriate constitutive basal, as well as, the inflammatory TNFα-induced NFκB transcriptional activity in ovarian cancer cell lines. Also surprisingly is that only loss of IKKβ and not AKT1 inhibits the Wnt3α- induced induction of β-catenin transcriptional activity. The inhibition of these two transcription factor and cytokines pathways that are known to promote carcinogenesis by AKT1 and IKKβ loss in ovarian cancer cells may offer the foundation for future studies on therapeutics targeting these pathways.
A.

B.

C.
Fig. 14. The effect of AKT1 or IKKβ loss on the NFκB and β-catenin transcriptional activation in the A2780 ovarian cancer cell line. A. NFκB Electrophoretic Mobility Shift Analysis (EMSA) of A2780 ovarian cancer cell line and AKT1- and IKKβ- knockdown clones and pools showing reduced TNF-α -induced NFκB DNA binding in the IKKβ knockdown cell lines compared to the parental line. Nuclear extracts were prepared at time 0 (control samples, C) and 30 minutes after treatment with TNF-α (treated samples, T). B. NFκB-dependent promoter activity in SKOV-3 ovarian cancer parental control cell line and AKT1- and IKKβ- knockout pools and clones. Cells were transiently co-transfected with a NFκB-dependent promoter reporter construct. After 24 hours, the cells were either unstimulated (C) or stimulated with TNF-α (T). The cells were harvested 4-6 hrs later and luciferase activity was determined. Data is expressed as mean normalized luciferase activity ± SD, n=3. C. β-catenin-dependent promoter activity in A2780 parental and knockdown clones showing reduced basal and Wnt3a-induced activities in all IKKβ – knockdown clones compared to the parental line. Cells were transiently transfected with pOT luciferase reporter construct and then stimulated with Wnt3a. Cells were harvested 24 hrs later and luciferase activity was determined. Data is expressed as mean Luciferase activity ± SD, n=3. L = control samples (treated with L-cell conditioned media). W = Wnt3a treated samples. Parent or A2780 = parental control cell line, A2780 AKT1p1 = A2780 AKT1 knockdown pool 1, A2780 AKT1c2 = A2780 AKT1 knockdown clone 2, A2780 AKT1c13 = A2780 AKT1 knockdown clone 13, A2780 IKKB1p1 = A2780 IKKβ knockdown pool 1, A2780 IKKB1c3 = A2780 IKKβ knockdown clone 3, A2780 IKKB1c8 = A2780 IKKβ knockdown clone 8.
**Specific Aim 2:** To examine the role of AKT1 and IKKβ in ovarian cancer cell proliferation, anchorage-independent growth, migration, and invasion

The loss of AKT1 and IKKβ had a dramatic effect on the NFκB and β-catenin oncogenic transcription factor pathways in ovarian cancer cells. As these two oncogenic transcription factor pathways control the expression of many genes that regulate the tumorigenic traits of cancer cells, we sought to further examine the effect of their loss on the oncogenic properties of ovarian cancer cells.

1. **Effects of AKT1 and IKKβ loss on the proliferation of A2780 ovarian cancer cells**

We first examined the consequence of AKT1 or IKKβ deficiency on the proliferation of the A2780 ovarian cancer cells. The growth of A2780 parental line and AKT1 and IKKβ- knockdown cell lines were assessed by using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega). This cell proliferation assay is based on the cellular conversion of a tetrazolium salt ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt or MTS) into a formazan product. The formazan absorbs at a characteristic wavelength, which can be read on a standard ELISA plate reader. Absorbance is directly proportional to the number of living cells in culture. The cells were plated at 5000/ well on 96-well plates. The next day the cells were treated with either control, TNF-α, L, or Wnt3a containing medium. The proliferation of the cells was evaluated after 24, 48 and 72 hours (Fig. 15A, B. C).

No significant differences in cell proliferation were observed for any cell line either treated with plain medium (as control) or plain medium plus TNFα or those treated
with L-cell conditioned medium (control) and Wnt3a-cell conditioned medium for all three timepoints studied. However, we observed differences in proliferation between the A2780 parent and the AKT1 and IKKβ knockdown cell lines, which demonstrated a slower rate of proliferation when measured at 48 and 72 hrs after the treatments (corresponding to 3 and 4 days after plating).

Next, to measure cellular number directly we also performed direct counting cells on the Guava PCA System using the Guava ViaCount Assay. This assay is a direct cell counting assay using flow cytometry and is based on a combination of two fluorescent nucleic acid dyes. The automated assay identifies cells while excluding debris, and distinguishing live from dead and apoptotic cells. Assay results include total cell count, and cell counts and percentages for both viable and apoptotic cells. A2780 parental and knockdown cell lines were plated in plain media and counted after 24, 48, 72 hrs. In this assay, we observed significant differences in cell number in the both the AKT1 and IKKβ-knockdown cell lines only after 72hrs (Fig. 16 A, B, C) as compared the the parental A2780 cells. The data that cellular proliferation of the A2780 ovarian cancer cell AKT1 and IKKβ knockdown lines is slowed between 48 and 72 hours may indicate that targeting this pathway could potentially impede ovarian cancer cell growth. However, given the fact that we have not observed differences in the first 24 or 48 hours after plating in cell number in these cells, we will use 24-48 hours as a maximum time point for future experiments so that it is not a factor for assays which rely on cell number performed with these cells.
Fig. 15. Cellular proliferation of A2780 parental and AKT1 and IKKβ knockdown cell lines. Cellular proliferation of A2780 parental and AKT1 and IKKβ knockdown cell lines was measured after 24 (A), 48 (B), and 72 (C) hours of treatment with plain medium, medium containing 20 ng/ml TNFα (TNF), L-cell (L) medium or Wnt3a (Wnt3a) medium. Cells were plated in 96-well plates and treated the next day with plain, TNFα, L-cell or Wnt3a medium. Cell proliferation was assessed by CellTiter 96® AQueous One Solution Cell Proliferation Assay at the indicated time period. All data are expressed as mean Absorbencies ± SD, n=3. PAR = parental A2780 cell line, AKT1p1 = A2780 AKT1 knockdown pool 1, AKT1c2 = A2780 AKT1 knockdown clone 2, IKKBp1 = A2780 IKKβ knockdown pool 1, IKKB1c8 = A2780 IKKβ knockdown clone 8. C.
Fig. 16. Cellular proliferation of A2780 parental and AKT1 and IKKβ knockdown cell lines. Cell number of A2780 parental and AKT1- and IKKβ-knockdown cell lines at 24 (A), 48 (B) and 72 (C) hours after plating. All data are expressed as mean Cell Number ± SD, n=3. * = P < .001 and ** = P < .0001
2. Effects of AKT1 and IKKβ loss on the proliferation of SKOV-3 ovarian cancer cells

We next examined the consequence of AKT1 or IKKβ deficiency on the proliferation of the SKOV-3 ovarian cancer cells. Similar proliferation assays were performed for the SKOV-3 parental and knockdown lines. The growth of SKOV-3 parental line and AKT1 and IKKβ- knockdown cell lines was assessed by using the CellTiter 96® AQueous One Solution Cell Proliferation Assay. The cells were plated at 5000/ well on 96-well plates. The next day the cells were treated with either control, TNF-α, L, or Wnt3a containing medium. The proliferation of the cells was evaluated after 24, 48 and 72 hours (Fig. 17A, B, C).

No significant differences in cell proliferation were observed for any cell line either treated with plain medium (as control) or plain medium plus TNFα or those treated with L-cell conditioned medium (control) and Wnt3a-cell conditioned medium for all three timepoints studied. However, we observed differences in proliferation between the SKOV-3 parent and some of the knockdown cell lines. The AKT1 clone 10 and the IKKβ clone 14 demonstrated a slower proliferation rate at all timepoints studied, whereas the IKKβ pool 1 has a similar or faster proliferation rate as compared to the parent line.

Next, we also performed direct counting cells on the Guava PCA machine using the Guava ViaCount Assay. SKOV-3 parental and knockdown cell lines were plated in plain media and counted after 24, 48, 72 hrs (Fig. 18A, B, C).
A.

B.

C.
Fig. 17. Cellular proliferation of SKOV-3 parental and AKT1 and IKKβ knockdown cell lines. Cellular proliferation of SKOV-3 parental and AKT1 and IKKβ knockdown cell lines was measured after 24 (A), 48 (B), and 72 (C) hours of treatment with plain (plain) medium, medium containing 20 ng/ml TNFα (TNF), L-cell (L) medium or Wnt3a (Wnt3a) medium. Cells were plated in 96-well plates and treated the next day with plain, TNFα, L-cell or Wnt3a medium. Cell proliferation was assessed by CellTiter 96® AQueous One Solution Cell Proliferation Assay at the indicated time period. All data are expressed as mean Absorbencies ± SD, n=3. Par= SKOV3 parental control cell line, SKOV-3 AKT1 p1 = SKOV-3AKT1 knockdown pool 1, SKOV-3 AKT1 c10 = SKOV-3AKT1 knockdown clone 10 SKOV-3 IKKB1p1 = SKOV-3 IKKβ knockdown pool 1, SKOV-3 IKKB1c14 = SKOV-3 IKKβ knockdown clone 14,
Fig. 18. Cellular proliferation of SKOV-3 parental and AKT1 and IKKβ knockdown cell lines. Cell number of SKOV-3 parental and AKT1- and IKKβ-knockdown cell lines at 24 (A), 48 (B) and 72 (C) hours after plating. All data are expressed as mean Cell Number ± SD, n=3. * = P ≤ .01 and ** = P ≤ .001
In this assay, we observed significant differences in cell number in the both the AKT1 and IKKβ-knockdown cell lines as compared the parental SKOV-3 cells only after 72hrs, with the exception of SKOV-3 AKT1 c10 which had a significant difference at 24 hrs (Fig. 18A, B, C). The data that cellular proliferation of the SKOV-3 ovarian cancer cell AKT1 and IKKβ knockdown lines is slowed between 48 and 72 hours gives further proof that targeting this pathway could potentially impede ovarian cancer cell growth. However, given the fact that we have not observed differences in the first 24 or 48 hours after plating in cell number in these cells except SKOV-3 AKT1 c10, we will use 24-48 hours as a maximum time point for future experiments so that it is not a factor for assays which rely on cell number performed with these cells.
3. Loss of AKT1 but not IKKβ inhibits anchorage independent growth in SKOV-3 and A2780 ovarian cancer cells

We next examined the consequence of AKT1 or IKKβ deficiency on the tumorigenic characteristic of anchorage independent growth of the A2780 and SKOV-3 ovarian cancer cells.

In order to determine the tumorigenic property of anchorage-independent cell growth, parental cells as well as IKKβ− and AKT1 -knockdown cells were plated in soft agar and incubated for 14 days. Staining of colonies allowed for quantification of growing colonies of cells.

For SKOV-3 cells, anchorage-independent colony formation is inhibited in all the AKT-1 knockdown pools and clonal cell lines. (Specifically for SKOV-3 AKT1 p1 we observed a decrease of 81.9% in colony formation, for SKOV-3 AKT1 c7 a decrease of 86.7% and for SKOV-3 AKT1 c10 a decrease of 83.9 %). Furthermore colony formation is reduced in 2 of the IKKβ knockdown clonal lines (Specifically, we observed a decrease of 23.9% for SKOV-3 IKKβ c8 and a decrease of 75.2% for SKOV-3 IKKβ c14) but not in the IKKβ knockdown pool or clone #13 (we observed an increase of 12.2% for SKOV-3 IKKβ p1 and a decrease of 2.5% for SKOV-3 IKKβ c13) (Fig. 19).
A.

B.

![Graph showing number of colonies for different cell lines.](image)
**Fig. 19. Loss of AKT1 but not IKKβ inhibits soft agar colony growth of SKOV-3 ovarian cancer cells.**

**A.** Soft agar colony formation. SKOV-3 parental and AKT- and IKKβ-knockdown cell lines suspended in 0.33% bactoagar growth medium and were incubated for 14 days at 37°C, 5% CO2. Fourteen days later, the plates were examined for colony formation indicative of anchorage-independent growth and stained with Crystal Violet. Pictures were taken and colonies were counted. The data shown are representative of three independent experiments with similar results. **B.** Number of colonies formed per 6-cm soft agar plate by SKOV-3 parental and AKT1- and IKKβ- knockdown cell lines. Graphical representation of the number of anchorage independent colonies formed (in duplicate) from the assays shown in fig. 19A. Data are expressed as mean Number of Colonies per 6-cm soft agar plate, n=2. The data are representative of three independent experiments with similar results. SKOV3 parent = parental control cell line, SKOV-3 AKT1 p1 = SKOV-3AKT1 knockdown pool 1, SKOV-3 AKT1 c7 = SKOV-3 AKT1 knockdown clone 7, SKOV-3 AKT1 c10 = SKOV-3 AKT1 knockdown clone 10, SKOV-3 IKKB1p1 = SKOV-3 IKKβ knockdown pool 1, SKOV-3 IKKB1c8 = SKOV-3 IKKβ knockdown clone 8, SKOV-3 IKKB1c13 = SKOV-3 IKKβ knockdown clone 13, SKOV-3 IKKB1c14 = SKOV-3 IKKβ knockdown clone 14
For A2780 ovarian cancer cells, colony formation is significantly reduced in the AKT-1 pool (we observed a 59.0% decrease in colony formation) and clone #2 (74.4% decrease in colony formation). However, AKT-1 clone #13 only had moderate reduction (decrease of 20.4% in colony formation) (Fig. 20A, B).

However like the results for the SKOV-3 cell lines, IKKβ knockdown did not have an inhibitory effect on soft agar colony formation. A2780 IKKβ pool had a 25.0% increase in colony formation and the clone #8 a 14.7% increase compared to the parent, while clone #3 was similar to the parent line in colony formation (Fig. 20).

This data indicate that loss of the either AKT1, but not IKKβ, dramatically inhibits the oncogenic trait of anchorage independent growth in ovarian cancer cell lines. The inhibition of the ability to survive and grow in the absence of anchorage to the extracellular matrix and neighboring cells is one of the most important oncogenic properties of cancer cells. Therefore these studies may offer the foundation for future studies on therapeutics which may target AKT to inhibit this important oncogenic property.
A.

A2780 parent

A2780 Akt pool

A2780 Akt p1

A2780 Akt c2

A2780 Akt c13

A2780 B1 pool

A2780 B1 c3

A2780 B1 c8

B.

Number of colonies

A2780 parent

A2780 Akt1 p1

A2780 Akt1 c2

A2780 Akt1 c13

A2780 IKK1 B1 p1

A2780 IKK1 c3

A2780 IKK1 c8
Fig. 20. Loss of AKT1 but not IKKβ inhibits soft agar colony growth of A2780 ovarian cancer cells.  

A. Soft agar colony formation. A2780 parental and AKT- and IKKβ-knockdown cell lines suspended in 0.33% bactoagar growth medium and were incubated for 7 days at 37°C, 5% CO2. One week later, the plates were examined for colony formation indicative of anchorage-independent growth and stained with Crystal Violet. Pictures were taken and colonies were counted. The data shown are representative of three independent experiments with similar results.  

B. Number of colonies formed per 6-cm soft agar plate by A2780 parental and AKT1- and IKKβ- knockdown cell lines. Graphical representation of the number of anchorage independent colonies formed (in duplicate) from the assays shown in figure 20A. Data are expressed as mean Number of Colonies per 6-cm soft agar plate, n=2. The data are representative of three independent experiments with similar results and shows an inhibition of colony formation in the A2780 AKT1 knockdown cell lines compared to the parental line. A2780 parent = A2780 parental control cell line, A2780 AKT1p1 = A2780 AKT1 knockdown pool 1, A2780 AKT1c2 = A2780 AKT1 knockdown clone 2, A2780 AKT1c13 = A2780 AKT1 knockdown clone 13, A2780 IKKB1p1 = A2780 IKKβ knockdown pool 1, A2780 IKKB1c3 = A2780 IKKβ knockdown clone 3, A2780 IKKB1c8 = A2780 IKKβ knockdown clone 8.
4. Both AKT1 and IKKβ loss inhibit the migration and invasion of the A2780 ovarian cancer cells

We next examined the consequence of AKT1 or IKKβ deficiency on the tumorigenic characteristics of cellular migration and invasion of the A2780 ovarian cancer cells. The migration and invasive potential of A2780 parental line and AKT1 and IKKβ- knockdown pools was measured by standard membrane (BD BioCoat Control Inserts) and matri-gel invasion assays (BD BioCoat Matrigel Invasion Chambers). The control inserts are an 8 µm pore size membrane which allows passage of migrating cells from the upper to the lower chamber while the matrigel invasion chambers are the control inserts that uniformly coated with BD Matrigel Matrix. The matrigel serves as a reconstituted basement membrane in vitro. This matrix provides a true barrier to non-invasive cells while presenting an appropriate protein structure for invading cells to penetrate before passing through the membrane. Therefore, you can measure migration of cells through the 8 µm pores of the control inserts and invasion of cells through the matrigel barrier of the matrigel invasion chambers.

For each cell line the experiment was done in triplicates, using 100000 cells per chamber. The migratory and invasive capacity of the cell lines was assessed after 24 hours.

Both migration and invasion are inhibited for the A2780 AKT1 and IKKβ- knockdown pools compared to the parent. The migratory capacity of the AKT1
knockdown pool was inhibited by 51% compared to the parent, and migratory capacity of the IKKβ- knockdown pool was inhibited by 44% compared to the parent line (fig. 21A). The invasive capacity of the AKT1 knockdown pool was inhibited by 82% compared to the parent, and the invasive capacity of the IKKβ knockdown pool was inhibited by 73% compared to the parent line (fig. 21B). Even taking the reduced migration into consideration, the AKT1 and IKKβ A2780 knockdown cell lines have reduced invasion capacity as indicated by the ratio of the number of cells capable of invasion to the number of cells capable of migrating (fig. 21C).

These results indicate that loss of either AKT1 or IKKβ causes a significant loss of both the tumorigenic migratory and invasive capacity of A2780 cells.
A.

![Bar chart showing the number of migrated cells for A2780 parent, A2780 Akt 1 p1, and A2780 IKKB1 p1. The bars are labeled with asterisks (*) and double asterisks (**) indicating statistical significance.](image)

B.

![Bar chart showing the number of invaded cells for A2780 parent, A2780 Akt 1 p1, and A2780 IKKB1 p1. The bars are labeled with double asterisks (**) indicating statistical significance.](image)

C.

![Bar chart showing the ratio of invaded to migrated cells for A2780, AKT1P1, and IKKB1P1. The bars are labeled in shades of gray, with A2780 having the highest ratio.](image)
Fig. 21. Loss of AKT1 and IKKβ inhibits migration and invasion of the A2780 ovarian cancer cell line. 

A. Graphical representation of cells that migrated through BD BioCoat Control Inserts for A2780 parental and AKT1 and IKKβ- knockdown pools. Loss of either AKT1 or IKKβ significantly decreased the migration of A2780 cells. Data are expressed as mean Number of Migrated Cells ± SD, n=3. 

B. Graphical representation of cells that invaded through BD BioCoat Matrigel Invasion Chambers for A2780 parental and AKT1 and IKKβ- knockdown pools. Loss of either AKT1 or IKKβ significantly inhibited the invasiveness through Matrigel of A2780 cells. Data are expressed as mean Number of Invaded Cells ± SD, n=3. 

C. Invasion capacity of A2780 cell lines = average number of cells that invaded/ migrated of parental and AKT1 and IKKβ- knockdown pools. * = P ≤ .01 and ** = P ≤ .001. A2780 parent or A2780 = A2780 parental control cell line, A2780 AKT1p1 = A2780 AKT1 knockdown pool 1, A2780 IKKB1p1 = A2780 IKKβ knockdown pool 1.
5. Loss of AKT1 and IKKβ inhibits the invasion but does not affect migration of the SKOV-3 ovarian cancer cells

We next examined the consequence of AKT1 or IKKβ deficiency on the tumorigenic characteristics of cellular migration and invasion of the SKOV-3 ovarian cancer cells. The migration and invasive potential of the SKOV-3 parental cell line and the AKT1 and IKKβ knockdown pools was measured using the standard membrane and matrigel invasions assays as before.

For each cell line the experiment was done in triplicates, using 10000 cells per chamber. The migratory capacity of the cell lines was assessed after 6 hours. Migration of the SKOV-3 AKT1 and IKKβ knockdown pools is similar to the parent line (fig. 22A).

Although there is no deficiency in migration, the invasive capacity of the AKT1 knockdown pool was inhibited by 71% compared to the parent, and the invasive capacity of the IKKβ knockdown pool was inhibited by 38% compared to the parent line (fig. 22B, C).

These results indicate that loss of either AKT1 or IKKβ causes only a loss of the invasive capacity in SKOV-3 cells, without affecting migration of these cells.

This data indicate that loss of the either AKT1 or IKKβ dramatically inhibits the oncogenic trait of invasion in ovarian cancer cell lines. In contrast, the oncogenic trait of migration was inhibited only in the A2780 but not the SKOV-3 ovarian cancer cell lines when AKT1 or IKKβ is lost. The differences between the two ovarian cancer lines with respected to migration indicates that these two ovarian cancer lines may differ dramatically in the pathways regulating cellular migration. The inhibition of the ability to
migrate and invade through extracellular matrix is an important oncogenic property of metastatic cancer cells. Therefore, these studies may offer the foundation for future investigation on therapeutic interventions which may target AKT and IKKβ to inhibit these important metastatic properties.
A.

![Bar chart showing number of migrated cells in SKOV-3, SKOV-3 Akt1p1, and SKOV-3 IKKB1p1].

B.

![Bar chart showing number of invaded cells in SKOV-3, SKOV-3 Akt1p1, and SKOV-3 IKKB1p1].

C.

![Bar chart showing ratio of invaded to migrated cells in SKOV3, AKT1P1, and IKKB1P1].
**Fig. 22. Loss of AKT1 and IKKβ inhibits the invasion, but not the migration, of the SKOV-3 ovarian cancer cell line.**

A. Graphical representation of cells that migrated through BD BioCoat Control Inserts for SKOV-3 parental and AKT1 and IKKβ-knockdown pools. Loss of either AKT1 or IKKβ does not affect the migration of SKOV-3 cells. Data are expressed as mean Number of Migrated Cells ± SD, n=3.

B. Graphical representation of cells that invaded through BD BioCoat Matrigel Invasion Chambers for SKOV-3 parental and AKT1 and IKKβ-knockdown pools. Loss of either AKT1 or IKKβ significantly inhibited the invasiveness through Matrigel of SKOV-3 cells. Data are expressed as mean Number of Invaded Cells ± SD, n=3.

C. Invasion capacity of SKOV-3 cell lines = average number of cells that invaded/migrated of parental and AKT1 and IKKβ-knockdown pools. * = P ≤ .01 and ** = P ≤ .001. SKOV3 = SKOV3 parental control cell line, SKOV-3 AKT1 p1 = SKOV-3AKT1 knockdown pool 1, SKOV-3 IKKB1p1 = SKOV-3 IKKβ knockdown pool 1.
6. SPARC expression is increased in AKT1 and IKKβ- knockdowns of both SKOV-3 and A2780 ovarian cancer cell lines

We next examined one of the interesting candidate genes that we identified through a preliminary microarray analysis as a gene whose expression levels were induced as a consequence of AKT1 or IKKβ deficiency in the SKOV-3 ovarian cancer cell line. We focused on SPARC (secreted protein, acidic and rich in cysteine) as it has been identified as a tumor suppressor in ovarian, neuroblastomas and colorectal cancer but a tumor promoter in other types of cancers such as melanomas and gliomas (Tai et al, 2008).

SPARC was identified through preliminary micro-array analysis as a gene whose expression was induced upon inhibition of the AKT/IKK signaling pathway. To verify the expression of SPARC in our knockdown lines of the SKOV-3 and A2780 ovarian cancer cells, we plated the parental and AKT1 and IKKβ knockdown cell lines on 10cm culture plates. Before the plates reached confluency, the cells were collected and lysed. Total protein extracts from each sample and used for western blot analysis (fig. 23A). Also, we plated the SKOV-3 and A2780 parental and AKT1 and IKKβ knockdown pools on 10cm culture plates, treated with serum free media and after 24 hrs we collected conditioned media and concentrated it using Amicon Ultra Concentrators. 50μg of protein from each sample were used for western blot analysis (fig. 23B). Our results show that SPARC expression is increased in the total protein extracts from SKOV-3 AKT1c10 and IKKB1p1 knockdown cell lines compared to the parent line and in all the A2780
AKT1 and IKKβ knockdown cell lines compared to the parent line. Also, SPARC expression is increased in the conditioned media collected from the SKOV-3 IKKβ knockdown pool compared to the parent line and in the conditioned media collected from the A2780 AKT1 and IKKβ knockdown pools compared to the parental line.

These results of SPARC expression increasing with loss of AKT1 and IKKβ positively correlate with the decreased invasion that we have observed in the knockdown pools of these ovarian cancer cells and could be at least in part responsible for this less tumorigenic phenotype seen in these cells.

In summary, the data obtained for specific aim #2 indicate that loss of AKT1 but not IKKβ in ovarian cancer cell lines can inhibit the oncogenic trait of anchorage independent growth while loss of either AKT1 or IKKβ can promote the expression of an ovarian cancer tumor suppressor gene, as well as, inhibit ovarian cancer cell proliferation and the oncogenic properties of migration and invasion.
A.

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<th>SKOV-3</th>
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<td>AKT1c10</td>
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SPARC

β actin

B.

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SPARC
Fig. 23. Loss of AKT1 and IKKβ induces expression of SPARC. A. Western Blot Analysis for expression of SPARC in the SKOV-3 and A2780 parental and knockdown cell lines. Cells were lysed and total protein extracts were analyzed for SPARC levels using the respective primary antibody. β-actin was used as loading control. Upper panel: parent = SKOV3 parental control cell line, SKOV-3 AKT1 p1 = SKOV-3 AKT1 knockdown pool 1, SKOV-3 AKT1 c10 = SKOV-3 AKT1 knockdown clone 10, SKOV-3 IKKB1p1 = SKOV-3 IKKβ knockdown pool 1, SKOV-3 IKKB1c14 = SKOV-3 IKKβ knockdown clone 14. Lower panel: parent = A2780 parental control cell line, A2780 AKT1p1 = A2780 AKT1 knockdown pool 1, A2780 AKT1c2 = A2780 AKT1 knockdown clone 2, A2780 IKKB1p1 = A2780 IKKβ knockdown pool 1, A2780 IKKB1c8 = A2780 IKKβ knockdown clone 8. B. Western Blot Analysis for expression of SPARC in the concentrated conditioned media collected form SKOV-3 and A2780 parental and AKT1- and IKKβ- knockdown pools. Left panel: parent = SKOV3 parental control cell line, SKOV-3 AKT1 p1 = SKOV-3 AKT1 knockdown pool 1, SKOV-3 IKKB1p1 = SKOV-3 IKKβ knockdown pool 1. Right panel: parent = A2780 parental control cell line, A2780 AKT1p1 = A2780 AKT1 knockdown pool 1, A2780 IKKB1p1 = A2780 IKKβ knockdown pool 1.
**Specific Aim 3:** To assess the roles of AKT1 and IKKβ in ovarian cancer chemotherapeutic resistance.

The loss of AKT1 and IKKβ had a dramatic effect on the NFκB and β-catenin oncogenic transcription factor pathways in ovarian cancer cells. As these two oncogenic transcription factor pathways control the expression of many genes that regulate the resistance to apoptosis of cancer cells, we sought to further examine the effect of their loss on the chemotherapeutic responsiveness of ovarian cancer cells.

1. **Loss of AKT1 or IKKβ increases chemotherapeutic sensitivity to Paclitaxel in A2780 ovarian cancer cells**

   We first examined the consequence of AKT1 or IKKβ deficiency on the responsiveness of the A2780 ovarian cancer cells to the chemotherapeutics paclitaxel and cisplatinum.

   The A2780 parental line was treated with different concentrations of Paclitaxel (PCTX) and Cisplatinum (CPT) for 48 hrs to determine the IC$_{50}$ (inhibitory dose to achieve 50% cell death) (data not shown). After 48 hrs treatment, cells were assayed using the CellTiter 96® AQ$_{	ext{ueous}}$ One Solution Cell Proliferation Assay. A decrease in A2780 cell viability of around 50% was observed with treatments of 30nM PCTX or 5 μM CPT. Based on these results, we decided to use these two drug concentrations to test the sensitivity of the AKT1 and IKKβ knockdown A2780 cell lines as compared to the A2780 parental line.
The A2780 parental as well as the AKT1 and IKK\(\beta\) knockdown A2780 cells were plated at 5000 cells/well on 96-well plates. The next day the cells were treated with either plain medium (vehicle control), PCTX (15 and 30nM) and CPT (5 and 10\(\mu\)M). The effect of the drugs on cell proliferation was evaluated after 48 hours (Fig. 24). The decrease in cell viability observed was calculated and is shown in table no. 4. The knockdown A2780 cell line pools of either AKT-1 or IKK\(\beta\) are significantly more sensitive to PCTX compared to the A2780 parental cell line. In contrast, the knockdown of AKT1 or IKK\(\beta\) did not significantly alter the sensitivity of A2780 cells to CPT at a dose of 5mM CPT but surprisingly actually decreased the sensitivity of the A2780 cells to the higher dose of 10mM CPT.

**Table 4. Decrease in cell viability in A2780 parent, AKT1 and IKK\(\beta\) knockdown pools after 48hrs treatment with PCTX and CPT.**

<table>
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<tr>
<th></th>
<th>% decrease in cell viability after treatment with PCTX 15nM</th>
<th>% decrease in cell viability after treatment with PCTX 30nM</th>
<th>% decrease in cell viability after treatment with CPT 5(\mu)M</th>
<th>% decrease in cell viability after treatment with CPT 10(\mu)M</th>
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<td>A2780</td>
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<tr>
<td>A2780 B1p1</td>
<td>47%</td>
<td>52%</td>
<td>22%</td>
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2. TGFBI expression is increased when AKT1 and IKKβ are lost in A2780 ovarian cancer cells.

We next examined another of the interesting candidate genes that we identified through the preliminary microarray analysis as a gene whose expression levels were induced as a the consequence of AKT1 or IKKβ deficiency in the SKOV-3 ovarian cancer cell line and also codes for secreted protein as does the other gene we studied, SPARC. We focused on TGFBI (transforming growth factor beta induced) also known as b-igH3 as it has been identified as a gene that modulates responsiveness to paclitaxel. (Ahmed et al, 2007).

TGFBI was identified through preliminary micro-array analysis as a gene whose expression was induced upon inhibition of the AKT/IKK signaling pathway. To verify if our knockdown pools of the A2780 ovarian cancer cell line express more TGFBI than the parental line, we plated the A2780 parental and AKT1 and IKKβ knockdown pools on 10cm culture plates, treated with serum free media and after 24 hrs we collected conditioned media and concentrated it using Amicon Ultra Concentrators. 50μg of protein from each sample were used for western blot analysis (fig.24B). Our results show that TGFBI expression is increased in the conditioned media collected from the knockdown pools compared to the parent line.
A.

B.
Fig. 24. Loss of AKT1 or IKKβ increase the sensitivity of A2780 cells to paclitaxel.

A. Drug sensitivity of A2780 parental and AKT1- and IKKβ- knockdown pools. The graphical representations show that the A2780 AKT-1 or IKKβ knockdown pools are significantly more sensitive to PCTX compared to the parental cell line. Cells were incubated for 48 hrs without treatment and treated with PCTX at 15 and 30 nM and CPT at 5 and 10μM. Cell number was assessed by CellTiter 96® AQueous One Solution Cell Proliferation Assay at the indicated time period. All data are expressed as mean Absorbencies ± SD, n=3. PCTX15, 30 = sample treated with 15nM respectively 30nm PCTX. CPT5, 10 = samples treated with 5μM respectively 10μM CPT. A2780 = parental A2780 cell line, A2780 AKT pool = A2780 AKT1 knockdown pool 1, IKKB1p1 = A2780 IKKβ knockdown pool 1.

B. Western Blot Analysis (with the respective antibody) of equal protein concentrations of concentrated conditioned media collected form A2780 parental and AKT1- and IKKβ- knockdown pools. parent = parental A2780 cell line, AKT1 = A2780 AKT1 knockdown pool 1, IKKB1 = A2780 IKKβ knockdown pool 1.
These results of TGFBI expression increasing with loss of AKT1 and IKKβ positively correlate with the increased sensitivity to PCTX that we have observed in the knockdown pools of these ovarian cancer cells and could be at least in part responsible for the increased sensitivity of the A2780 ovarian cancer cells to this widely used ovarian cancer chemotherapeutic
3. Loss of AKT1 or IKKβ increased apoptosis in response to Paclitaxel in A2780 ovarian cancer cells

Next, we wanted to see whether the increased sensitivity of the AKT1 and IKKβ knockdown A2780 cell line pools to PCTX treatments is due to an increase in apoptosis. A2780 parental cells were plated in 10cm culture plates and treated with 30nM PCTX for 24, 48, 72hrs or left untreated (as control). We assessed the number and percentage of live, dead and apoptotic cells on each plate by using the Guava ViaCount assay. The results are shown below (fig.25). PCTX 30nM treatment induces apoptosis in the A2780 parental cell line. The increase in apoptosis observed was calculated and is shown below in table 5.

Table 5. Increase in cell apoptosis in A2780 parent untreated and treated with PCTX 30 nM for 24, 48, 72 hrs.

<table>
<thead>
<tr>
<th>Sample Description</th>
<th>% of apoptotic cells</th>
<th>% of live cells</th>
<th>% of dead cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2780 control</td>
<td>1.8</td>
<td>93.4</td>
<td>4.8</td>
</tr>
<tr>
<td>A2780 PCTX 24 hrs</td>
<td>4.5</td>
<td>84.8</td>
<td>10.7</td>
</tr>
<tr>
<td>A2780 PCTX 48 hrs</td>
<td>11.2</td>
<td>70.5</td>
<td>18.3</td>
</tr>
<tr>
<td>A2780 PCTX 72 hrs</td>
<td>13.2</td>
<td>65.3</td>
<td>21.5</td>
</tr>
</tbody>
</table>
**Fig. 25. Paclitaxel causes apoptosis of A2780 ovarian cancer cells.** Percentage of live, apoptotic and dead cells in cultures of A2780 parental untreated (control) and treated with PCTX 30 nM for 24, 48, 72 hrs. Cells were grown in 10 cm culture plates and treated with PCTX 30 nM. Cells were collected and analyzed by the Guava ViaCount Assay according to manufacturer’s protocol.
We further tested the sensitivity of the AKT1 and IKKβ- knockdown cell line pools to PCTX in comparison to the A2780 parental cell line. We plated the cells on 10cm culture dishes and the next day treated them with PCTX 30nM for 48 hrs. The control plates were left untreated in plain medium (vehicle control). The experiment was done with triplicates of each sample. We assessed the number and percentage of live, dead and apoptotic cells on each plate by using the Guava ViaCount assay. The results are shown below (fig. 26). Our results show an increased percentage of both apoptotic and dead cells in the PCTX 30nM treated AKT1 (apoptotic/ dead = 6.70%/10.07% in treated samples vs.0.57%/ 1.27% in untreated samples) and IKKβ- (apoptotic/ dead = 4.91%/12.79% in treated samples vs.0.43%/ 1.06% in untreated samples) A2780 knockdown pools plates compared to the parent line (apoptotic/ dead = 7.26%/15.66% in treated samples vs.0.73%/ 2.43% in untreated samples). We also performed a Guava TUNEL Assay on these samples to assess the percentage of apoptotic cells also showing a significant increase in apoptosis following the treatment with Paclitaxel in the knockdown pools compared to the parent line (results shown below in table 6).

<table>
<thead>
<tr>
<th></th>
<th>Fold increase in apoptotic cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2780 PCTX vs. control</td>
<td>5.08</td>
</tr>
<tr>
<td>A2780 AKT1p1 PCTX vs. control</td>
<td>26.14</td>
</tr>
<tr>
<td>A2780 IKKB1p1 PCTX vs. control</td>
<td>21.46</td>
</tr>
</tbody>
</table>
**Fig. 26. Loss of AKT or IKKβ increases apoptosis of A2780 ovarian cancer cells in response to paclitaxel.** Graphical representation of total, viable, apoptotic and dead cells on plates in cultures treated with PCTX 30 nM for 48hrs vs untreated of A2780 parental, AKT1 and IKKβ knockdown pools. Cells were grown in 10 cm culture plates, treated with PCTX 30 nM and 48 hrs later they were trypsinized and counted using Guava ViaCount Assay (Guava Technologies) on a Guava PCA machine according to manufacturer’s protocol Data is expressed and mean Number of Cells ± SD, n=3. ctrl = control samples (untreated), PCTX = samples treated with PCTX 30nM for 48 hrs.
PCTX is known to also cause cell cycle arrest in addition to apoptosis in cancer cells. Therefore, we next performed a cell cycle analysis on the control and 30nM PCTX treated parental, the AKT1 and IKKβ known-down A2780 ovarian cancer cell lines. Cells were grown in 10 cm culture plates, synchronized for 36 hrs in serum free media, and then either untreated (vehicle control) or treated with 30 nM PCTX for 24 hrs. Cells were collected, fixed and stained using Guava Cell Cycle Assay (Guava Technologies) on a Guava PCA machine according to manufacturer’s protocol. Treatment of cells with 30 nM PCTX caused a G2/M arrest as expected in all the A2780 parental, AKT1 and IKKβ knockdown cell lines but loss of AKT1 or IKKβ did not increase significantly change the G2/M cell cycle arrest (fig. 27).

These data indicate that loss of either AKT1 or IKKβ in A2780 ovarian cancer cells lead to an increased sensitivity to PCTX chemotherapeutic treatment which is likely linked to the decreased survival pathway signaling in these cells causing increased apoptosis and cell death.
Fig. 27. Loss of AKT1 or IKKβ does not alter G2/M cell cycle arrest of A2780 ovarian cancer cell in response to paclitaxel. Cell cycle analysis for cultures treated with 30 nM PCTX vs untreated of A2780 parental, AKT1 and IKKB1 knockdown pools. Cells were grown in 10 cm culture plates, synchronized for 36 hrs in serum free media and then treated with 30 nM PCTX for 24 hrs. Cells were collected, fixed and stained using Guava Cell Cycle Assay according to manufacturer’s protocol. Paclitaxel induces a G2/M cell cycle arrest in all cell lines, and inhibits G0/G1 to S transition in the A2780 knockdown pools. Data is expressed as mean Percentages in G0/G1, S or G2/M phases ± SD, n=3.
4. Loss of AKT1 or IKKβ increases chemotherapeutic sensitivity to Cisplatinum in SKOV-3 ovarian cancer cells

We next examined the consequence of AKT1 or IKKβ deficiency on the responsiveness of the SKOV-3 ovarian cancer cells to the chemotherapeutics paclitaxel and cisplatinum. The SKOV-3 parental line was treated with different concentrations of PCTX and CPT for 48 hrs to determine the IC$_{50}$. After 48 hrs treatment, cells were assayed using the CellTiter 96® AQueous One Solution Cell Proliferation Assay. We observed a decrease in cell viability of 58% when using 10nM or 12.5 nM PCTX and about 63% for 15nM PCTX. Furthermore, a 27% decrease at 20 μM CPT and 62% decrease for 25 μM CPT in cell viability was observed (data not shown). Based on these results, we decided to further test the sensitivity of the SKOV-3 AKT1 and IKKβ knockdown pools using drug concentrations of 5 and 10 nM PCTX and 12.5 and 25 μM of CPT (Fig. 28).

The decrease in cell viability observed was calculated and is shown in table 7.

Table 7. Decrease in cell viability in SKOV-3 parent, AKT1 and IKKβ knockdown pools after 48hrs treatment with PCTX at 5 and 10 nM and CPT at 12.5 and 25μM.

<table>
<thead>
<tr>
<th></th>
<th>% decrease in cell viability after treatment with PCTX 5 nM</th>
<th>% decrease in cell viability after treatment with PCTX 10 nM</th>
<th>% decrease in cell viability after treatment with CPT 12.5 μM</th>
<th>% decrease in cell viability after treatment with CPT 25 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>SKOV-3</td>
<td>35%</td>
<td>46%</td>
<td>36%</td>
<td>42%</td>
</tr>
<tr>
<td>SKOV-3Akt1p1</td>
<td>27%</td>
<td>40%</td>
<td>52%</td>
<td>79%</td>
</tr>
<tr>
<td>SKOV-3IKKB1p1</td>
<td>28%</td>
<td>48%</td>
<td>49%</td>
<td>58%</td>
</tr>
</tbody>
</table>
**Fig. 28. Loss of AKT1 or IKKβ increases the sensitivity of SKOV-3 cells to cisplatinum.** Drug sensitivity testing of SKOV-3 parental and AKT1- and IKKβ-knockdown pools. The graphical representations show that the SKOV-3 AKT-1 or IKKβ knockdown pools are significantly more sensitive to CPT compared to the parental cell line. Cells were incubated for 48 hrs without treatment and treated with PCTX at 5 and 10 nM and CPT at 12.5 and 25μM. Cell number was assessed by CellTiter 96® AQueous One Solution Cell Proliferation Assay at the indicated time period. All data are expressed as mean Absorbencies ± SD, n=3. PCTX 5, 10 = samples treated with 5nM, 10nM PCTX respectively. CPT 12.5, 25 = samples treated with 12.5μM, 25μM CPT respectively. SKOV-3 = SKOV3 parental control cell line, SKOV-3 AKT1 pool = SKOV-3 AKT1 knockdown pool 1, SKOV-3 IKKβ pool1 = SKOV-3 IKKβ knockdown pool 1.
Knockdown cell line pools of either AKT-1 or IKKβ are significantly more sensitive to CPT as compared to the SKOV-3 parental cell line while no significant increase or even a decrease in sensitivity to PCTX was observed for the SKOV-3 knockdown cell lines as compared to the parental cell line.
5. Loss of AKT1 or IKKβ increased apoptosis in response to Cisplatinum in SKOV-3 ovarian cancer cells

We further tested the sensitivity of the AKT1 and IKKβ- knockdown cell line pools to CPT in comparison to the SKOV-3 parental cell line. We plated the cells on 10cm culture dishes and the next day treated them with CPT at 25μM for 48 hrs. The control plates were left untreated in plain medium (vehicle control). The experiment was done with triplicates of each sample. We assessed the number and percentage of live, dead and apoptotic cells on each plate by using the Guava ViaCount assay. The results are shown below (fig. 29). However, this assay did not demonstrate any significant changes in the apoptotic or dead population of cells. We next performed a Guava TUNEL Assay on these samples to assess the percentage of apoptotic cells and these results showed a significant increase in apoptosis following the treatment with Cisplatinum in the knockdown pools compared to the parent line (results shown below in table 8).
**Fig. 29. Apoptosis of SKOV-3 ovarian cancer cells in response to cisplatinum.**

Graphical representation of total, viable, apoptotic and dead cells on plates in cultures treated with CPT at 25μM for 48 hrs vs untreated of SKOV-3 parental, AKT1 and IKKB1 knockdown pools. Cells were grown in 10 cm culture plates, treated with CPT 25μM and 48hrs later were trypsinized and counted using Guava ViaCount Assay (Guava Technologies) on a Guava PCA machine according to manufacturer’s protocol. Data is expressed and mean Number of Cells ± SD, n=3. ctrl = control samples (untreated), CPT = samples treated with CPT 25μM for 48 hrs.
Table 8. Fold Increase in apoptotic cells in SKOV-3 parental, AKT1 and IKKβ knockdown pools untreated and treated with CPT 25 μM for 48 hrs.

<table>
<thead>
<tr>
<th></th>
<th>Fold increase in apoptotic cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>SKOV-3 CPT vs. control</td>
<td>10.02</td>
</tr>
<tr>
<td>SKOV-3 AKT1p1 CPT vs. control</td>
<td>32.60</td>
</tr>
<tr>
<td>SKOV-3 IKKB1p1 CPT vs. control</td>
<td>28.58</td>
</tr>
</tbody>
</table>

The data from the Guava TUNEL assay indicate that loss of either AKT1 or IKKβ in SKOV3 ovarian cancer cells leads to an increased sensitivity to CPT chemotherapeutic treatment which is likely linked to the decreased survival pathway signaling in these cells causing increased apoptosis and cell death.

In summary, the data obtained for specific aim #3 indicate that loss of either AKT1 or IKKβ can promote the expression of a gene that modulates responsiveness to paclitaxel, as well as, increase the sensitivity of A2780 ovarian cancer cell to PCTX but not CPT while increases the sensitivity of SKOV-3 cells to CPT but not PCTX. These data may be important for future targeting of these pathways as perhaps an adjuvant therapy in combination with these therapeutics.
CHAPTER 4

DISCUSSION AND FUTURE DIRECTIONS

Ovarian cancer is one of the most common gynecological malignancies in women, showing increased mortality rate and difficulties in treatment because of the diagnosis at advanced stages and due to the resistance to chemotherapeutics. The future for more effective treatment of ovarian cancer lies in earlier detection and better response to chemotherapy of these deadly tumors.

The AKT cell survival pathway is frequently activated in ovarian cancer in which elevated AKT1 activity (Sun et al, 2001) is often observed. Elevated AKT activity can be a major source of resistance to chemotherapy observed in many cancers. Amplification of PIK3CA (Shayesteh et al, 1999; Willner et al, 2007; Levine et al, 2005) or AKT2 (Bellacosa et al, 1995), for example, aberrantly increases AKT cell survival signaling. Also PTEN, a tumor suppressor and negative regulator of the AKT pathway, is frequently mutated in endometrioid but not serous or mucinous epithelial ovarian tumors (Obata et al, 1998). Previously, our laboratory discovered that the anti-apoptotic transcription factor, NFκB, was controlled by the AKT cell survival signaling pathway (Sizemore et al, 1999, 2002). Our previous results demonstrated that the transcriptional activity of NFκB is positively and negatively regulated by PI3K/AKT cell survival and the PTEN tumor suppressor pathway, respectively (Sizemore et al, 1999, 2002). The PI3K/AKT and PTEN pathways control NFκB transcriptional activity by regulating the ability of the
IKKs to phosphorylate the p65 subunit of NFκB (Sizemore et al, 1999). Our previous data indicated that the AKT/IKK pathway not only regulate NFκB but also regulates the activity of another important oncogenic transcription factor, β-catenin (Agarwal et al, 2005). Furthermore the concurrent regulation of both NFκB and β-catenin by the AKT/IKK pathway promotes angiogenic and metastatic gene expression in colorectal cancer (Agarwal et al, 2005). The PI3K/ AKT/ IKK signaling pathway controls many cellular oncogenic functions thus playing a critical role in many types of cancer. Therefore, targeting of this pathway in ovarian cancer could be used for future therapeutic interventions that may stem the high mortality associated with this understudied cancer.

Recent studies have demonstrated the clinical potential of synthetic small interfering RNAs (siRNAs) or short hairpin RNAs (shRNAs) in many diseases and cancers (Pushparaj et al, 2008). The concept of RNAi was originally introduced by Andrew Fire (Montgomery et al, 1998). RNAi represents a mechanism by which gene expression is silenced by preventing the transcription of specific target genes. The development of RNAi technology in the last decade has provided a valuable research tool, as the dsRNA introduced into cells can down-regulate gene expression of specific genes of interest. siRNAs (short interfering RNAs) have complementary nucleotide sequence to the target mRNA and the process leads to the degradation of mRNA, which prevents its translation into protein. In this study, we have used RNAi technology to specifically knockdown the expression of the AKT1 kinase and the IKKβ subunit of the IKK complex and determined the effects on the regulation of the NFκB and β-catenin
signal transduction pathways as well as on the tumorigenic traits and chemotherapeutic resistance in ovarian cancer cells.

We successfully identified several ovarian cancer lines in which the AKT and IKK pathways were activated and chose two, A2780 and SKOV-3, for our shRNA mediated inhibition of this pathway (Fig. 9 and 10) The shRNA constructs that we designed both efficiently and specially reduced the expression of either AKT1 or IKKβ in two human ovarian cancer cell lines (Fig.12). This allowed us to set up two ovarian cancer line models to further study the effects on the regulation of the NFκB and β-catenin signal transduction pathways as well as on the tumorigenic traits and chemotherapeutic resistance in ovarian cancer cells. As our shRNA designs for reducing AKT1 and IKKβ were so effective in tissue culture cells and strategies for the inhibition of cancer cell proliferation in tumor-bearing animals by tissue-specific shRNA delivery treatment are beginning to emerge (Ghatak et al, 2008), it is possible that they could be adapted to a lentivirus or other high efficiency delivery system for potential clinical applications to treat ovarian cancer in the future.

NFκB and β-catenin are two transcription factors important in regulating the expression of genes that control cell proliferation, cell survival and transformation. Our hypothesis is that aberrant activation of AKT and IKKs may cause the deregulation and inappropriate activation of both the NFκB and the β-catenin signal transduction pathways, which may lead to the development of ovarian cancer by promoting cellular transformation, uncontrolled cell division and resistance to apoptosis.
Our results show that inhibition of the AKT/IKK pathway can block the constitutive activation of the NFκB (Fig. 13 and 14). Loss of either IKKβ or AKT1 significantly inhibited both the inappropriate basal and TNFα-induced NFκB transcriptional activity and loss of IKKβ reduced TNF-α -induced NFκB DNA binding in The SKOV-3 and A2780 ovarian cancer cell lines (Fig. 13 and 14). This new result reinforces this previous concept first proposed by our group and then others demonstrating that this pathway is important in regulation the inflammatory and oncogenic NFκB transcription factor (Martin et al, 2000; Sakurai et al, 1999; Sizemore et al, 1999 and 2002; Wang et al, 1998 and 2000). Also, we found that IKKβ knockdown significantly inhibited both the basal and Wnt-stimulated β-catenin-dependent transcriptional activity in A2780 ovarian cancer cell line (Fig 14). The concurrent loss of β-catenin transcriptional activation with IKKβ knockdown is a novel finding and previously we and others had only identified IKKα, not IKKβ, as a positive regulator of β-catenin (Agarwal et al, 2005, Albanese et al, 2003). Our new data may indicate a common pathway in which AKT can activate both IKKα and IKKβ to control the activity of both NFκB and β-catenin but more work is needed. This data, together with other studies, may play an important role in understanding how deregulation of the AKT pathway can lead to aberrant activation of the NFκB and β-catenin pathways which in theory could be key in the development and progression of ovarian cancer.

Ovarian cancer is one of the most difficult cancers to detect and treat and it has the worst diagnosis among gynecological diseases because it is caught at advanced stages when metastases are already present. The in vivo tumorigenicity of cancer cells can be
somewhat predicted by their capacity to form colonies in an anchorage independent manner. The inhibition of the ability to survive and grow in the absence of anchorage to the extracellular matrix and neighboring cells is one of the most important oncogenic properties of cancer cells. Therefore as two important oncogenic transcription factor pathways were dramatically affected, we measured the ability of our ovarian cancer cell lines in which shRNA specifically reduced either AKT1 or IKKβ expression to grow in an anchorage independent environment by plating the cells in soft agarose. Our results showed that knockdown of AKT1, but not IKKβ, by shRNA inhibited anchorage independent growth in both SKOV-3 and A2780 ovarian cancer cells (Fig 19 and 20). Therefore, we predict that the AKT1 knockdown lines would form fewer tumors with a longer latency in xenograft experiments as compared to the parental lines. Thus like many other tumors the targeting of AKT1 in ovarian cancer cells seems to dramatically increase programmed cell death in response to growth of the ovarian cancer cells in the absence of extracellular matrix or anoikis (Simpson et al., 2008). This could be an important finding as future studies could be done to specifically assess the role of NFκB and β-catenin transcriptional activity and control by AKT1 in ovarian cancer cell anoikis.

Ovarian cancer cell migration and invasion are important steps in ovarian cancer progression, leading to metastasis formation. The current focus for researchers is to identify new potential treatments to target these processes. Understanding the involvement of the PI3K/ AKT/ IKK signaling pathway in ovarian cancer cell migration and invasion may potentially lead to a more effective therapeutic treatment. An important finding in our study is that knockdown of either AKT1 or IKKβ by shRNA inhibited both
migration and invasion of the A2780 ovarian cancer cells (Fig. 21), while knockdown of either AKT1 or IKKβ caused only an inhibition of the invasive capacity in SKOV-3 cells, without affecting migration of these cells (Fig. 22). Why the migration of SKOV-3 ovarian cancer cells is resistant to loss of AKT1 and IKKβ is of future interest and could lie in the oncogenic molecular mutations that differ between the two ovarian cancer lines.

The rate of cell proliferation can alter migration and invasion of cells. However, in this study we did not find differences in cell proliferation between the parental and knockdown pools at the time points used for the invasion and migration assays (Fig. 15, 16, 17 and 18), suggesting that cell proliferation does not play a significant role in the migration and invasion of these ovarian cancer cells. Our results that AKT and IKKβ promote cancer cell migration and invasion add further support to the current literature (Karin, 2008; Sheng et al, 2008) defining a critical role of both AKT and IKK in migration, invasion, and metastasis. Therefore future studies on determining the individual contributions of the NFκB and β-catenin pathways to AKT- and IKK-mediated metastatic properties would be of high interest especially as applied to ovarian cancer.

One consequence of AKT1 or IKKβ deficiency in our ovarian cancer cell line models was a dramatic induction of SPARC (Fig. 23) especially in the A2780 cells and it has been identified as a tumor suppressor in ovarian, neuroblastomas and colorectal cancer but a tumor promoter in other types of cancers such as melanomas and gliomas (Tai et al, 2008). SPARC, also known as osteonectin or BM-40, is an extracellular Ca^{2+} -binding matricellular glycoprotein that modulates interactions between cells and ECM
The classical function of SPARC was linked to ossified and mineralized tissues (as a Ca\(^{2+}\) - binding protein). Also, previous studies have showed SPARC’s involvement to tissue morphogenesis and in cellular differentiation. The role of SPARC in tumorigenesis is complex. SPARC has anti-adhesive and anti-proliferative functions, modulates angiogenesis and regulates the production and the organization of the extracellular matrix (Tai et al, 2008). SPARC modulates the microenvironment surrounding the tumors and has been shown to play different roles in different cancers. It has been reported that SPARC promotes adhesion, invasion and tumorigenicity in melanoma (Ledda et al, 1997) and glioma invasion (Schultz et al, 2002) and it increases motility and invasion of breast cancer cells (Briggs et al, 2002). SPARC functions as a tumor suppressor in ovarian cancer. However, the available data on how SPARC “normalizes” the ovarian tumor microenvironment in order to make it unfavorable to tumor growth is still limited. SPARC was shown to inhibit integrin-mediated adhesion and growth factor-stimulated survival signaling pathways in ovarian cancer (Said N. et al, March 2007). Also, SPARC has an anti-proliferative effect and induces apoptosis on ovarian cancer (Yiu GK et al, 2001). Overexpression of SPARC reduces LPA-induced proliferation, chemotaxis, and invasion of ovarian cancer cell lines (Said N et al, January 2007). SPARC down-regulates the VEGF – integrin - MMP axis, decreasing the levels and activity of bioactive lipids and therefore ameliorates the peritoneal tumor – induced inflammatory response (Said N et al, Oct 2007) and the absence of SPARC was shown to increase peritoneal ovarian carcinomatosis (Said N et al, Dec 2005). All these findings suggest that SPARC plays a key role in ovarian cancer cell motility, invasiveness, and
metastasis. Our novel finding in this study is that our A2780 AKT1 and IKKβ knockdown cell lines showed an increased expression of SPARC in the total protein extracts and in the conditioned media collected from these cells (Fig 23). Over-expression of SPARC in these knockdown cells seems to be in parallel with the reduced invasion and motility of these cells (Fig 21). Our data adds additional evidence that SPARC is an ovarian cancer tumor and metastasis suppressor in ovarian cancer and agrees with the previous studies that reported reduced peritoneal carcinomatosis as well as anti-adhesive and anti-invasive functions for SPARC. In light of recent studies demonstrating that SPARC negatively regulates MMP levels and reduces the levels and activity of bioactive lipids thus playing a key role in the ovarian cancer microenvironment, the molecular mechanisms controlling SPARC expression in our ovarian cancer cell model systems would be important. These future studies could lead to more information on how SPARC is regulated and functions in ovarian cancer. These studies would be necessary to determine whether SPARC would a possible therapeutic target given that SPARC can play a tumor promoting role in other types of cancers.

One of the most challenging aspects of chemotherapy in ovarian cancer which impacts the long-term efficacy of the treatment is drug resistance. Understanding the mechanisms by which certain cancer cell populations become resistant is important so that in the future we can develop efficient anticancer therapies. Two of the chemotherapeutic drugs most commonly used for the treatment of ovarian cancer are Paclitaxel and Cisplatinum. One of the objectives of our studies was to evaluate the effects of the interruption of the AKT/ IKK signaling pathway in the A2780 and SKOV-3
cell lines on the chemotherapeutic resistance of these ovarian cancer cells. *Hu et al (2002)* have shown *in vivo* that the effect of combination therapy with LY294002 and Paclitaxel is significantly greater than the effect of each treatment alone on tumor growth and ascites formation. Our experimental results show here that the loss of either AKT1 or IKKβ in A2780 ovarian cancer cells increased chemotherapeutic sensitivity to Paclitaxel (Fig. 24 and Table #4) and also that loss of either AKT1 or IKKβ increased apoptosis in response to Paclitaxel in A2780 ovarian cancer cells (Fig. 25, 26, 27 and Table #5 and #6). Previous studies have shown that paclitaxel response can be modulated by extracellular matrix proteins. Transforming growth factor beta induced (TGFBI); also known as b-big H3 or keratoepithelin, is an ECM protein that mediates specific sensitization to paclitaxel by inducing stabilization of microtubules via integrin-mediated signaling pathways (*Ahmed et al, 2007*). Our data shows that TGFBI expression is increased in the of AKT1 and IKKβ knockdown pools of the A2780 ovarian cancer cell line (Fig. 24), positively correlating with the increase in sensitivity to Paclitaxel in these knockdown cell lines. TGFBI is induced by targeted of AKT1 and IKKβ in the A2780 cells and may play a role in the increased paclitaxel-induced apoptosis observed in this ovarian cancer cell line. The potential molecular mechanisms by which knockdown of AKT1 and IKKβ lead to increased expression of TGFBI needs further study and explanation. Of interest would be the contribution of the NFκB and β-catenin transcription factor pathways in controlling the expression of this gene. However, we have confirmed a novel role of the AKT1 and IKKβ in controlling both paclitaxel-induced apoptosis and the expression of this important gene, TGFBI, that modulates
paclitaxel responsiveness by directly targeting them by RNA interference. Our study indicates that inhibition of AKT1 and IKKβ can be used as an adjuvant therapy with paclitaxel for better treatment of ovarian cancer. Recently, another study has confirmed the involvement of AKT2 in paclitaxel induced apoptosis by regulating surviving expression in ovarian cancer cell lines (Weng et al, 2008). Our study gives a new perspective to the field by investigating the role that AKT1 plays in ovarian cancer, as most of the existing data today refers to AKT2. Taken together, these results suggest that in ovarian cancer, different AKT isoforms may modulate paclitaxel response through different cellular target proteins and further exploration is required for a better understanding of the mechanisms by which this chemotherapeutic drug acts on malignant cells.

Cisplatin is a very potent antitumor agent, displaying clinical activity against a wide variety of solid tumors. Its cytotoxic mode of action is mediated by its interaction with DNA and the formation of DNA adducts, primarily intrastrand crosslink adducts (Brunton et al). This DNA damage activates several signal transduction pathways, including those involving AKT, ATR, p53, p73, and MAPK, and culminates in the activation of apoptosis (Brunton et al). DNA damage-mediated apoptotic signals, however, can be attenuated, and the resistance that ensues is a major limitation of cisplatin-based chemotherapy. Mabuchi et al (2004) have shown that inhibition of NFκB activity by using BAY 11-7085 (an IκBα phosphorylation inhibitor) combined with cisplatinum significantly reduced the volume of ascites, intra-abdominal dissemination and the proportion of apoptotic cells compared to cisplatinum alone in an in vivo study.
using CAOV-3 ovarian cancer cells in athymic nude mice model. Hayakawa et al (2000) have reported that inhibition of AKT by overexpression of kinase-deficient AKT sensitizes human ovarian cancer cells to cisplatinum. Our data also showed that the loss of either AKT1 or IKKβ increased chemotherapeutic sensitivity to cisplatinum in SKOV-3 ovarian cancer cells (Fig. 28, 29 and Table #7 and #8). The observed decrease in cell viability is attributed to increased apoptosis in response to cisplatinum in the AKT1 and IKKβ knockdown cell lines compared to parent in SKOV-3 ovarian cancer cells. Our study demonstrates that inhibition of AKT1 and IKKβ can potentially be used as an adjuvant therapy with cisplatinum for treatment of ovarian cancer.

In conclusion, our results suggest that AKT1 and IKKβ are two major molecules involved in ovarian cancer progression. Finding new targeting approaches for these molecules and their downstream effectors could provide a successful approach for screening and treating patients with ovarian cancer. The PI3K/ AKT/ IKK signaling pathway seems to play a key role in ovarian cancers as in many other cancers, by targeting several cellular proteins involved in cell survival and apoptosis and also angiogenesis. AKT and IKKα (but not IKKβ) have been previously shown to play important roles in activation of both NFκB and β-catenin in colorectal cancer. Our results confirm that in ovarian cancer cells both AKT1 and IKKβ are important in the positive regulation of the NFκB and β-catenin transduction pathways, leading to expression of several target genes, among these being several secreted proteins (SPARC, TGFBI, MMPs) that play key roles in ovarian cancer cell adhesion, invasion, anchorage independent growth and sensitivity to chemotherapeutics. Our findings and the modified
NFκB and β-catenin signaling pathways in ovarian cancer cells are illustrated in Fig. 30 (A and B).

For future directions, I believe it would be interesting to evaluate the effect of the AKT1 and IKKβ knockdown in the A2780 and SKOV-3 ovarian cancer cell lines in an *in vivo* ovarian tumor xenograft system (as all our studies so far have been done *in vitro*). For these assays, the tumorigenic and metastatic potential of the control and knockdown cell lines will be measured by subcutaneous injection in the flanks of nude mice and tumor growth and metastasis will be evaluated by standard analysis. Also, similar experiments will be conducted to evaluate the therapeutic ability of cisplatin and paclitaxel to inhibit the tumorigenic and metastatic potential of the control and knockdown cell lines (subcutaneous injection in the flanks of nude mice followed by i.v. injection of cisplatin and paclitaxel or vehicle starting 7 days after tumor inoculation). Compared to the *in vivo* studies, *in vitro* experiments done so far have limitations as they do not take into consideration drug - plasma protein interactions and metabolism of the drug. Finally, the PI3K/ AKT/ IKK pathway has been implicated in tumor angiogenesis through regulation of VEGF signaling. VEGF is known to increase vascular permeability and plays an important role in tumor neovascularization. VEGF was shown to play an important role in ascites formation and in subcutaneous tumor growth in immunodeficient mice injected with SKOV-3 cancer cells (*Mesiano et al, 1999*). Therefore, another direction for future research is to evaluate the anti-angiogenic effects of the AKT1 and IKKβ knockdown in our engineered cell lines in both the *in vitro* and *in vivo* models.
Ovarian Cancer

Fig. 30. A. NFκB activation in our ovarian cancer cell system. B. Wnt signaling pathway in our ovarian cancer cell system.


American Cancer Society www.cancer.org


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SIGMA Cisplatin Product Information

SIGMA Paclitaxel Product Information


