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## DOWNREGULATION OF PAX2 SUPPRESSES OVARIAN CANCER CELL GROWTH

huijuan song

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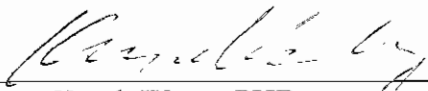
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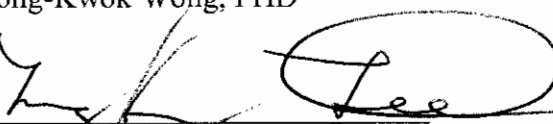
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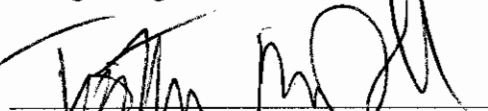
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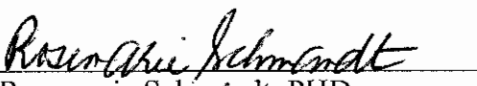
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**DOWNREGULATION OF PAX2 SUPPRESSES OVARIAN CANCER CELL  
GROWTH**

**A**

**DISSERTATION**

**Presented to the Faculty of**

**The University of Texas**

**Health Science Center at Houston**

**and**

**The University of Texas**

**M. D. Anderson Cancer Center**

**Graduate School of Biomedical Sciences**

**In Partial Fulfillment of the**

**Requirements of the Degree of**

**DOCTOR OF PHILOSOPHY**

**By**

**Huijuan Song, MD, MS**

**Houston, Texas**

**August 2011**

## DEDICATION

This dissertation is dedicated to my parents, Shuangli Song and Chunhua Yan for their eternal love and trust. I want to cite Celine Dion and Warren Diane's *because you loved me* and dedicate these to my parents.

“For all those times you stood by me,  
For all the truth that you made me see,  
For all the joy you brought to my life,  
For all the wrongs that you made right,  
For every dream you made come true,  
For all the love I found in you,  
I'll be forever thankful.  
You're the one who held me up and never let me fall.  
You're the one who saw me through it all.  
You were my strength when I was weak.  
You were my voice when I couldn't speak.  
You were my eyes when I couldn't see.  
You saw the best there was in me.  
Lifted me up when I couldn't reach.  
You gave me faith because you believed in me.  
I'm everything I am because you loved me.  
You gave me wings and made me fly.  
You touched my hand, I could touch the sky.  
I lost my faith; you gave it back to me.  
You said no star was out of reach.  
You stood by me and I stood tall.  
I had your love I had it all.  
I'm grateful for each day you gave me.  
Maybe I do not know that much.  
But I know this much is true.  
I was blessed because I was loved by you (1).”

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I would like to thank my mentor, Dr. Kwong-Kwok Wong, for supporting me through my whole PHD study, especially when I was down. This scene has been burned in my memory.

“When I am down and my soul so weary;  
When troubles come and my heart burdened be,  
Then, I am still and wait here in the silence,  
Until you come and sit a while with me (2).”

You are always there when I need you and your support is a gift that god has vouchsafed me. I would also like to thank my former mentor, Dr. Zhen Zhen, for her inspiration and trust. She is the one who opened my eyes and brought forth the beautiful world in front of me.

I would like to thank all of my committee members who have guided me along this path to success: Dr. Paul Chiao, Dr. Mong-Hong Lee, Dr. Timothy McDonnell, Dr. Samuel Mok, Dr. Rosemarie Schmandt, Dr. Michael Van Dyke, Dr. Kwong-Kwok Wong, Dr. Dihua Yu and Dr. Wei Zhang. You are all my teachers. In china, we compare teacher to the candle who conflagrates itself and lightens others. I think nothing is more suitable than Brendan Graham and Josh Groban’s *you raise me up* to describe my teachers.

“You raise me up, so I can stand on mountains;  
You raise me up to walk on stormy seas;  
I am strong when I am on your shoulders;  
You raise me up to more than I can be (2).”

I would like to thank all the past and present members in Dr. Wong's laboratory for their friendship and help in the past several years. I would also like to thank my friends for their encouragement and support. Your companionships are the reasons for me to insist. "I'll always look back as I walk away and this memory will last for eternity (3)." I will dedicate Carpenters' *top of the world* to my colleagues and friends.

"Everything I want the world to be  
Is now coming true especially for me  
And the reason is clear  
It's because you are here  
You're the nearest thing to heaven that I've seen  
There is only one wish on my mind  
When this day is through I hope that I will find  
That tomorrow will be just the same for you and me  
All I need will be mine if you are here (4)."

At last, I would like to thank my families for their believing in and encouraging me to be the best.

# **DOWNREGULATION OF PAX2 SUPPRESSES OVARIAN CANCER CELL GROWTH**

Publication No. \_\_\_\_\_

Huijuan Song, MD, MS

Supervisory Professor: Kwong-Kwok Wong, PHD

*PAX2* is one of nine *PAX* genes regulating tissue development and cellular differentiation in embryos. *PAX2* promotes cell proliferation, oncogenic transformation, cell-lineage specification, migration, and survival. Unattenuated *PAX2* has been found in several cancer types. We therefore sought to elucidate the role of *PAX2* in ovarian carcinomas. We found that *PAX2* was expressed in low-grade serous, clear cell, endometrioid and mucinous cell ovarian carcinomas, which are relatively chemoresistant compared to high grade serous ovarian carcinomas. Four ovarian cancer cell lines, RMUGL (mucinous), TOV21G (clear cell), MDAH-2774 (endometrioid) and IGROV1 (endometrioid), which express high-levels of *PAX2*, were used to study the function of *PAX2*. Lentiviral shRNAs targeting *PAX2* were used to knock down *PAX2* expression in these cell lines. Cellular proliferation and motility assays subsequently showed that *PAX2* stable knockdown had slower growth and migration rates. Microarray gene expression profile analysis further identified genes that were affected

by PAX2 including the tumor suppressor gene *G0S2*. Reverse phase protein array (RPPA) data showed that PAX2 knockdown affected several genes that are involved in apoptosis, which supports the fact that downregulation of PAX2 in PAX2-expressing ovarian cancer cells inhibits cell growth. We hypothesize that this growth inhibition is due to upregulation of the tumor suppressor gene *G0S2* via induction of apoptosis. PAX2 represents a potential therapeutic target for chemoresistant PAX2-expressing ovarian carcinomas.



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## **ABBREVIATION**

ANXA1	Annexin A1
BCL2	B Cell Lymphoma Protein 2
BCL-XL	B Cell Lymphoma-Extra Large
ChIP	Chromatin-Immunoprecipitation
DHRS2	Dehydrogenase/Reductase Member 2
EGF	Epidermal Growth Factor
EGFR	EGF Receptor
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
FPR2	Formyl Peptide Receptor 2
FPRL-1	Formyl Peptide Receptor-Like 1
G0S2	G0/G1 Switch 2
GDNF	Glial-cell-line Derived Neurotrophic Factor
GPCR	G-protein Coupled Receptor
HMGCS1	3-Hydroxy-3-MethylGlutaryl-Coenzyme A Synthase 1
HOSE	Human Ovarian Surface Epithelium
HSPA8	Heat Shock 70kD Protein 8
IGF	Insulin-like Growth Factor
IHC	Immunohistochemical
IL-1	Interleukin 1



IP3	Inositol Triphosphate
JNK	c-Jun N-terminal Kinase
LMP	Low Malignant Potential
LXA4	Lipoxin A4
MAPK	Mitogen Activated Protein Kinase
MEF	Mouse Embryonic Fibroblast
MYCBP	c-Myc Binding Protein
mTOR	Mammalian Target of Rapamycin
NF-KB	Nuclear Factor kappa B
PAX	Paired Box
PDGF	Platelet Derived Growth Factor
PS	Phosphatidylserine
PI3K	Phosphoinositide-3 Kinase
PIP2	Phosphatidylinositol bisphosphate
PPIA	Cyclophilin A
PPRE	PPAR-responsive element
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
shRNA	Small Hairpin RNA
siRNA	Small Interfering RNA
TNF $\alpha$	Tumor Necrosis Factor alpha
TNFR	TNF $\alpha$ Receptor
TP53	Tumor Protein 53

TRIB3

Tribbles Homolog 3

## **CHAPTER 1**

### **INTRODUCTION**

## **1.1 Ovarian cancer**

Ovarian cancer begins in the ovaries or fallopian tubes. It is the second most common gynecologic malignancy, and the most common cause of death among women who develop gynecologic cancers (5). It is also the fifth most common cause of cancer-related death in females in the United States (5). There were approximately 21,880 new cases and 13,850 deaths in 2010 (6). It is called a “silent killer” (7) because in the early stage, the symptoms are vague and non-specific (8). The obvious symptoms can only be found when the disease has advanced. Thus, the chance of complete cure or remission is poor.

Ovarian carcinomas are a heterogeneous group of neoplasms and are traditionally subclassified based on type and degree of differentiation (9). Primary ovarian tumors, whether benign or malignant, can arise from three broad types of cells: epithelial cells (the cells on the outer surface of the ovary); germ cells (the cells that produce ova); and sex cord-stromal cells (the connective tissue cells surrounding the germ cells and producing ovarian hormones, such as estrogen and progesterone) (10).

Epithelial tumors are the most common type of ovarian cancers which comprise 85-90% of primary ovarian cancers and 60% of all ovarian cancers. Primary peritoneal cancers can be epithelial ovarian cancer or relatively related to it because they all come from the epithelial lining of the ovary. Later onset is one of the characteristics of epithelial ovarian cancers, although they can occur in the early decades of life (11).

The next common types of ovarian cancers are sex cord-stromal ovarian cancers which are derived from the connective tissue of the ovary and form fibroma or

fibrosarcoma. Sex cord-stromal ovarian cancers can also be derived from hormone producing granulosa cells and form thecoma. This type of ovarian cancer accounts for 10% to 15% of ovarian tumors and can produce both female and male hormone. They are commonly seen in premenopausal females (11).

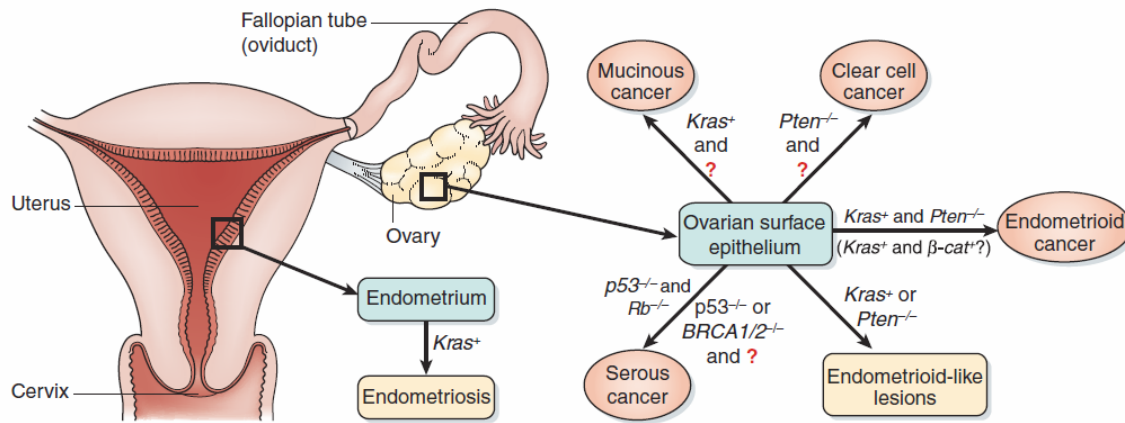
Germ cell ovarian cancers are derived from germ cells of the ovary and account for 5% of ovarian cancers. Most germ cell tumors are benign dermoid tumors or mature teratomas which have a rapid growth rate. Patients with germ cell ovarian cancers are relatively young and have more symptoms (11).

The most common morphological subtype of epithelial cell ovarian cancer is serous papillary ovarian cancer which is about 65% (12). Mucinous ovarian cancer accounts for 32% of the epithelial cell ovarian cancer (12, 13). The other less common histological subtypes are clear cell, endometrioid, transitional (Brenner), and undifferentiated ovarian cancer (Figure 1-1) (14).

**Figure 1-1. Genetic alterations of ovarian cancer.**

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In the development of ovarian cancer, the activation of oncogenes and inactivation of tumor suppressor genes play a key role. For example, the activation of Kras and inactivation of TP53 is the key player in the development of ovarian cancer. Some unknown secondary genetic changes are also involved in the ovarian cancer development (11).



Ovarian cancer treatment depends mainly on the subtype of the cancer and the stage of the disease. Other factors that are considered include the general state of the patient's health, whether the individual plans to have children, and other personal considerations. Conventional treatment for ovarian cancer is surgery. For some very early tumors (stage one, low grade disease), only the involved ovary and fallopian tube will be removed (known as "unilateral salpingo-oophorectomy," USO) (15) which is suitable for young women who wish to preserve their fertility. In more than 70% of cases, the tumor has disseminated beyond the ovaries when diagnosed and the combination of surgery and chemotherapy is necessary. First-line chemotherapy with platinum drugs (e.g. cisplatin and carboplatin) and taxanes (e.g. paclitaxel) yields a response rate of more than 80% (16); however, nearly all patients relapse. At the time of relapse, tumors can be re-challenged with platinum drugs and taxanes, with response rates proportional to the disease-free interval after the first treatment (17). In addition, chemotherapy can be used before surgery (known as neoadjuvant chemotherapy) to shrink the cancer and make it easier to be removed (18).

For low malignant potential (LMP, borderline) tumors, the ovary with the tumor and the fallopian tube is usually removed. Chemotherapy and radiation are not generally used for these tumors, although they may be used if the tumor recurs after surgery (19, 20). For early stage epithelial ovarian tumor, chemotherapy is commonly used, although radiation therapy can also be used. For stage III and stage IV cancers, chemotherapy is usually used after surgery (21, 22). High dose chemotherapy with stem cell rescue (i.e. bone marrow transplant) is used for women with recurrent and persistent cancer. This

approach has serious side effects and should be used cautiously (23). Thus, many targeted therapies using non-chemotherapy drugs to target specific cancer cells are in development. Unlike chemotherapy, targeted therapy spares normal cells, and may reduce the side effects of other therapies. Although targeting a transcription factor by small molecule is not very feasible, recent identification of an immunogenic HLA-A\*0201-binding T-cell epitope of the transcription factor PAX2 provides a promising view for cancer immunotherapy by targeting PAX2 (24).

The prognosis of ovarian cancer is affected by many factors, such as the stage of the disease, the type and size of the tumor, the patient's age and general health, and whether the cancer has just been diagnosed or has recurred. Over 90% of ovarian cancers are epithelial neoplasms which are classified as serous (30-70%), endometrioid (10-20%), mucinous (5-35%), clear cell (3-10%), and undifferentiated (1%) ovarian cancers, with the five-year survival rates of 20-35%, 40-63%, 40-69%, 35-50%, and 11-29%, respectively. The rest are germ cell or stromal cell ovarian cancers (12, 13). The non-serous ovarian cancers are relatively resistant to standard chemotherapy. It is important for us to find the proper therapeutic targets for these kinds of ovarian cancers. Kurman et al. (25) has divided ovarian cancers into Type I and Type II two groups. Type I tumors include low-grade micropapillary serous carcinoma, mucinous, endometrioid, and clear cell carcinomas. They are characterized by high genetic stability and mutations in *KRAS*, *BRAF*, *PTEN*, or *beta-catenin*(25). Type I tumors develop from LMP tumors which are generally confined to the ovary at diagnosis and grow slowly (25). Type II tumors include high-grade serous carcinoma, malignant



mixed mesodermal tumors (carcinosarcomas) and undifferentiated carcinomas. They are characterized by high genetic instability and mutations in *TP53*(25). Type II tumors when first detected are usually in advanced stages and appear as highly aggressive neoplasms, which lack well defined precursor lesions and grow rapidly (25). We found that PAX2 was expressed in low-grade serous, clear cell, endometrioid and mucinous cell ovarian carcinomas, which are relatively chemoresistant to the standard chemotherapy using platinum drugs (e.g. cisplatin and carboplatin) and taxanes (e.g. paclitaxel) (26, 27), compared with high grade serous ovarian carcinomas. This gives us further insight in the ovarian cancer treatment.

## **1.2 *PAX* gene family**
























*PAX* (paired box) genes were first described by Markus in 1986 (28) as a conserved sequence in the *Drosophila paired* and *gooseberry* genes. In 1988, Deutsch et al. (29) used the paired box domain from gooseberry as a probe and isolated the related gene sequences from the mouse genome, which is the first cloning of vertebrate *PAX* genes. Subsequently, eight murine (30) and nine mammal *PAX* genes (31) were identified. The nine members of human *PAX* gene family share a 384 base pair highly conserved DNA sequence, the paired box (32). Each human *PAX* gene is located on an entirely different chromosome which suggests that they act singularly and not in combination (33). Based on the structural similarity, sequence homology, the presence or absence of an octapeptide domain, and either a homeodomain or partial

homeodomain, the developmental *PAX* genes are divided into four subgroups (Figure 1-2) (34).

**Figure 1-2. PAX family members.**

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The nine members of human *PAX* gene family share a 384 base pair highly conserved DNA sequence, the paired box (32). Each human *PAX* gene is located on an entirely different chromosome which suggests that they act singularly and not in combination (33). Based on the structural similarity, sequence homology, the presence or absence of an octapeptide domain, and either a homeodomain or partial homeodomain, the developmental *PAX* genes are divided into four subgroups (34). *PAX* genes regulate tissue development and cellular differentiation in embryos by promoting cell proliferation, cell-lineage specification, migration and survival (35, 36).

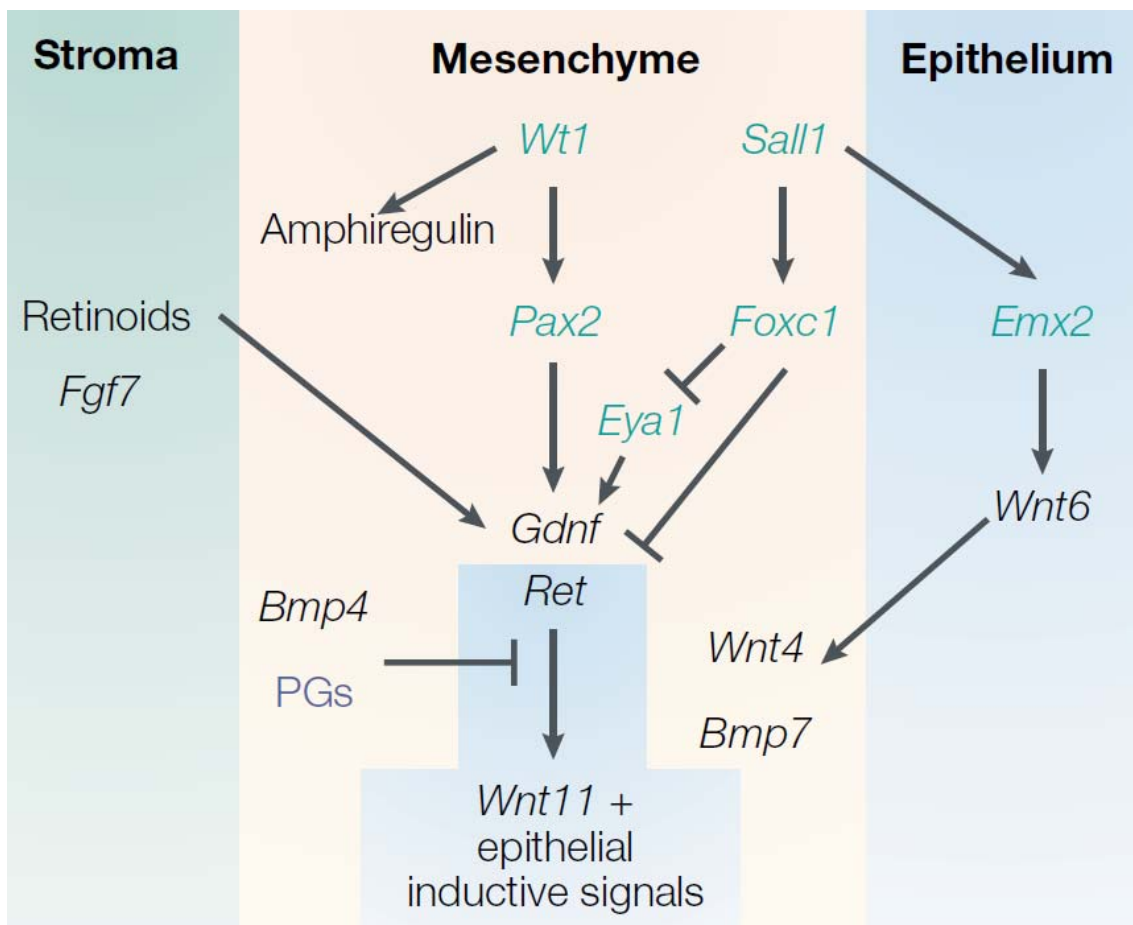
Gene	Human chromosome location	Group	Structural domains			Cancer contribution
			Paired domain	Octapeptide	Homeodomain	
<i>PAX3</i>	2q35	III				Tumour promoting
<i>PAX7</i>	1p36					
<i>PAX2</i>	10q24	II				Tumour promoting
<i>PAX5</i>	9p13					
<i>PAX8</i>	2q12					
<i>PAX4</i>	7q32	IV				Neutral or favourable
<i>PAX6</i>	11p13					
<i>PAX1</i>	20p11	I				Neutral or favourable
<i>PAX9</i>	14q12					

*PAX* genes regulate tissue development and cellular differentiation in embryos by promoting cell proliferation, cell-lineage specification, migration and survival (35, 36). They control the development of eyes, brain, CNS, vertebral column, neural crest, kidney, thyroid gland, immune system, pancreas, oviduct, vas deferens, epididymis, myogenic precursors of muscle tissue and a variety of other lineages (Figure 1-3) (35, 37-48). *PAX* genes regulate organogenesis and its mutations cause significant developmental abnormalities in a broad spectrum of organisms from flies to humans (49, 50). Their inactivation generally results in embryonal or neonatal death accompanied by striking developmental defects (38). In most cases, *PAX* gene expression attenuates when development is complete (34). Unattenuated *PAX* genes in adult tissues direct organ-specific regenerative events (51) and protect against stress-induced cell death (52). *PAX* genes are involved in stem-cell self-renewal, both during fetal development and in adult life which is important in tissue morphogenesis, regeneration and repair (35). Cancer cells may use the *PAX* gene pathway to undergo proliferation, stem-cell self-renewal and resistance to apoptosis, cell migration and invasion. These are some of the hallmarks of malignant growth as suggested by Hanahan and Weinberg (35, 53, 54). This indicates that *PAX* genes are proto-oncogenes (55) and deregulation of *PAX* genes contributes to the tumor formation (Figure 1-4) (56). Future research will be focused on the molecular mechanisms of *PAX* genes' function and their upstream regulators and downstream target genes which will contribute to both developmental processes and the molecular mechanisms underlying pathogenesis (55).

**Figure 1-3. PAX2 contributes to the initiation of kidney development.**

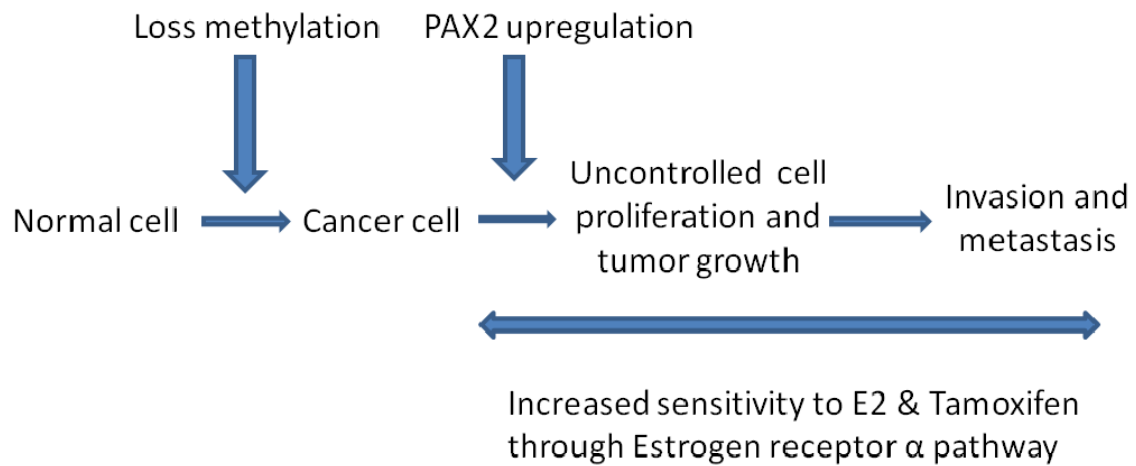
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PAX2 is regulated by WT1 in the initiation of kidney development. PAX2 also controls the initiation of kidney morphogenesis through controlling *Gdnf* expression (57).



**Figure 1-4. *PAX2* promotes endometrial carcinogenesis.**

*PAX2* reactivation leads to E2- and Tamoxifen-induced invasion and metastasis by driving E2- and Tamoxifen-mediated cell proliferation and tumor growth through estrogen receptor  $\alpha$  pathway (58-60).



### 1.3 PAX protein

*PAX* genes encode mRNAs ranging from 3.0 to 5.0 kb (61) except *PAX-5*, whose mRNA is 9.5-10 kb in length (62). The protein products of *PAX* genes, which are a family of transcription factors (35), vary from 360 to 480 amino acids in length (55). *PAX* proteins are characterized by the paired domain (PD), a conserved 128-amino acid DNA-binding motif at the amino-terminal end of the protein, which makes sequence-specific contacts with DNA (49). *PAX* proteins can also interact with DNA through the homeodomain located at the amino-terminus (39). The consensus DNA binding site for the paired domain is (G/T)T(T/C)(C/A)(C/T)(G/C)(G/C) (63) which is also the *PAX* protein binding site to the other proteins (50). *PAX* proteins are localized in the nucleus and bind to DNA *in vitro* (55, 64, 65). *PAX* proteins play an essential role in embryogenesis and organogenesis (66). They regulate cell proliferation, differentiation, self-renewal, resistance to apoptosis, migration of embryonic precursor cells, cell survival through target gene transactivation and specific differentiation programs (Figure 1-5) (50, 66). For example, *PAX2* and *PAX8* double mutants lack kidney formation completely (66, 67). *PAX* genes were found in tissue specific stem cells or progenitor cell populations which were found in a number of different adult tissues, such as blood, muscle, intestine and the skin. *PAX* proteins maintain the pluripotency of stem cell populations and cell-lineage specification during development by restricting lineage specification, resisting apoptosis and repressing terminal differentiation which may facilitate the development and progression of specific cancers (50). The anti-

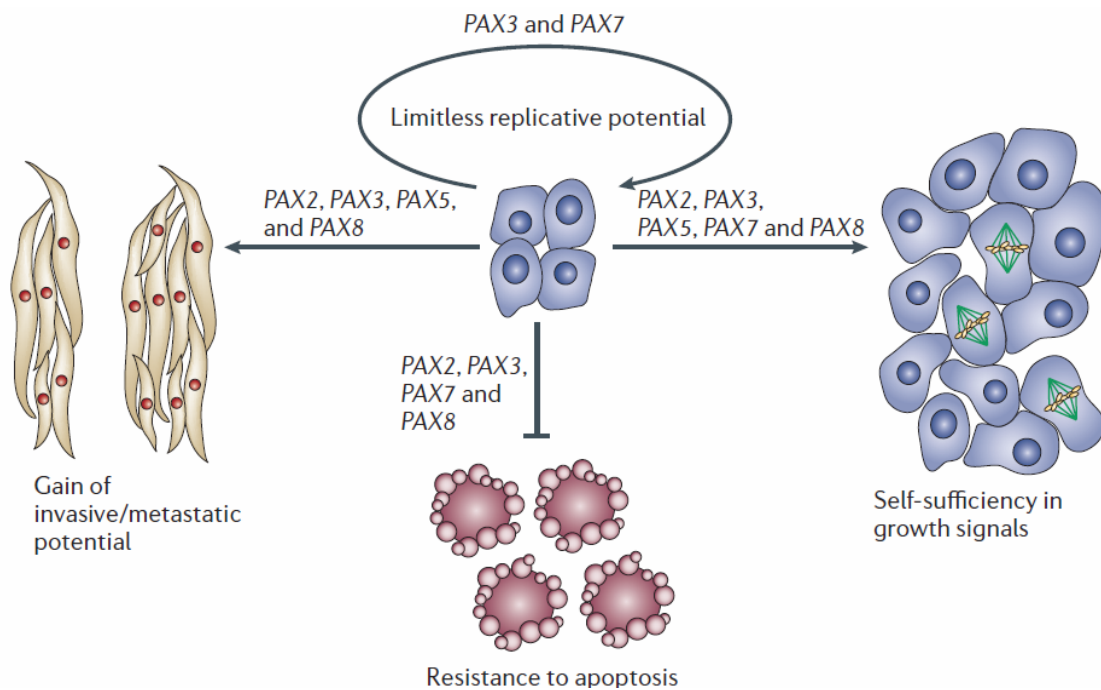
apoptotic and terminal differentiation repression functions of PAX protein are linked directly with cancer progression (50). PAX protein over-expression prompts malignant development instead of the initiating or transforming molecular event in tumor pathogenesis (50). For example, PAX2, PAX5, and PAX8 are expressed in multiple tumor cell lines, including renal cell carcinoma, and PAX2 is expressed in kidney cystic and hyperproliferative dysplastic diseases (50). Thus, PAX proteins may be useful as diagnostic markers and therapeutic targets for cancers. For instance, PAX2 immunostaining has been used to distinguish between metastatic ovarian serous papillary carcinoma and primary breast carcinoma (66). Transfection of tumor cells with PAX antisense oligonucleotides or RNA interference molecules is an effective gene-based cancer therapy because it induces cell apoptosis and reduces cell proliferation or migration (66). However, the role of PAX2 in ovarian cancer growth and development is not clearly understood. Further research will be focused on the regulators of *PAX* genes or target genes of PAX proteins (65) and the role of particular PAX proteins in cancer to find prognostic markers and/or potential anticancer therapeutic targets (50).



**Figure 1-5. PAX gene subgroups II & III promote tumor growth.**

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PAX gene subgroups II & III promote tumor formation and maintenance *via* mediating cells to acquire tumor characteristics (35).



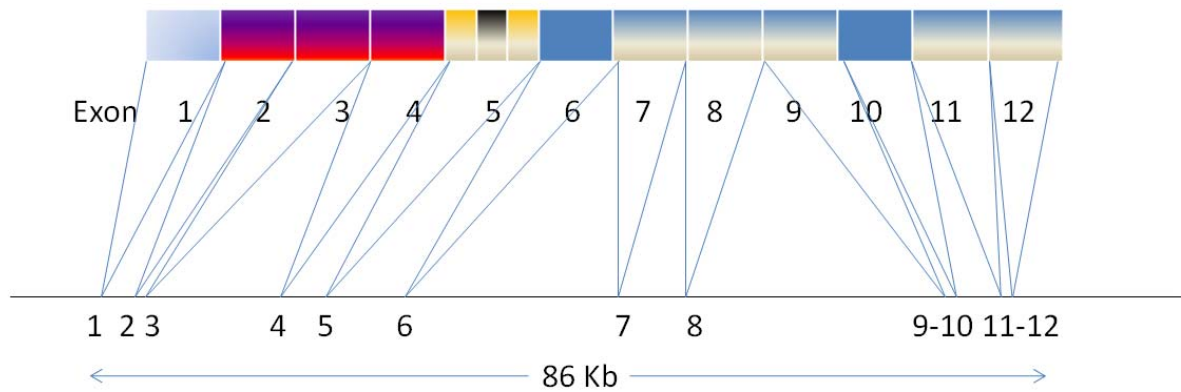
## 1.4 PAX2

*PAX2* belongs to the *PAX* gene subgroup II which is involved in embryonic development (68) and tumor growth (35, 69). Human *PAX2* gene is located on chromosome bands 10q24.3-10q25.1 (70) and composed of 12 exons which spans approximately 86 kb (71) and encodes 48-50 KDa *PAX2* protein (64) which is a transcription factor (46) and regulates the differentiation of urogenital system (37, 43), eyes (41), and central nervous system (46, 47) during the early development (Figure 1-6) (69, 72). Exons 1-4 are the paired box domain which is a highly conserved 128-amino acid DNA binding motif (71, 73, 74). Exon 5 is the octapeptide sequence which is another highly conserved repressor motif (47, 71) in the N-terminal to the homeodomain (39). Alternatively spliced exons include exon 6 which includes a 69-bp inserted sequence, exon 10 and exon 12 which contain an alternative acceptor splice site (71, 75). Exons 7-12 encode the carboxy-terminal portion of the *PAX2* protein which is essential for transcriptional activation of target genes and has strong activating and inhibitory domains (40). *PAX2* is abundantly expressed in the kidney (37, 43), ureter (42), eye (41), cochlear (44), pancreas (45) and central nervous system (47, 71) during embryogenic development and is important for their embryogenic development, morphogenesis and organogenesis (44-48). *PAX2* is expressed in proliferating cells during mesenchymal to epithelial transitions in these tissues; following mesenchymal to epithelial transition, *PAX2* expression is diminished or absent in the nascent and adult epithelial structures (35, 76). *PAX2* deficiency causes growth defects of kidney

hypoplasia, optic colobomas, and vesicoureteral reflux (77). Conversely, PAX2 overexpression is associated with epithelial overgrowth with cyst or tumor formation (39), such as cystic dysplastic kidneys, renal cell carcinomas, Wilms' tumors, and nephrogenic adenomas (48, 76) which indicates an undifferentiated or de-differentiated phenotype (78). High PAX2 expression in developing undifferentiated cells of the urogenital system indicates that PAX2 promotes cell differentiation and survival (73). PAX2 is important for the growth and survival of several cancers of urogenital origin (50) which indicates that *PAX2* maybe a proto-oncogene (79). The human *PAX2* gene is involved in the regulation of several genes, such as *WT1* (80), *N-myc* (81), *PAX5*, *PAX6*, *TP53*, and itself (46, 82) and it can be regulated by *FGF-8* and *PAX6*. For example, *WT1* transcriptionally represses the *PAX2* promoter and attenuates PAX2 expression during kidney morphogenesis (83); on the other hand, PAX2 represses WT1 expression in the presence of groucho/transducin-like enhancer proteins and transcriptionally activates the *WT1* promoter (84) in the absence of these proteins (85). Thus, there exists the cross-transcriptional control between these two genes, such that *PAX2* initially modulates the transcriptional activity of WT1 and at a later stage when a threshold level of WT1 protein is reached, the WT1 protein level in cells then represses *PAX2* transcription (40).

**Figure 1-6. Structure of the human *PAX2* gene.**

*PAX2* gene has 12 exons spanning 86 Kb. Exon 2, 3 and 4 are the paired box domain. Exon 5 contains octapeptide domain. Exon 6 and 10 are alternatively spliced exons (40).



## **1.5 G0S2**

G0S2 protein was first identified as a small basic nuclear phosphoprotein which was encoded by G0/G1 switch 2 (G0S2), one of the G0/G1 switch (G0S) genes that are differentially expressed during lymphocytes lectin-induced switch from G0 to G1 phases of the cell cycle (86). The expression of G0S2 is required to commit cells to enter the G1 phase of the cell cycle (87). G0S2 is highly expressed in adipose tissue, liver and heart. Its expression increases in response to glucose, insulin and ligands for the PPAR family of transcription factors, and decreases upon treatment with TNF $\alpha$  and  $\beta$ -adrenergic agonist (88, 89). G0S2 protein, a mitochondrial protein, specifically interacts with Bcl-2 and promotes apoptosis through preventing the formation of protective Bcl-2/Bax heterodimers (90). DNA methylation of the *G0S2* gene was significantly more frequent in squamous lung cancer than in non-squamous lung cancer (91). *G0S2* with a functional PPRE (PPAR-responsive element) in its promoter is a direct PPAR $\gamma$  and probable PPAR $\alpha$  target gene and may be involved in adipocyte differentiation (92).

## **1.6 Annexins**

The annexins are a superfamily of proteins which present in eukaryotic cells (93). Annexins are made up of a conserved  $\alpha$ -helical core domain which is constituted by four repeats of 60–70 amino acids each, attached to a unique N-terminal region which is expelled from the core domain on calcium binding (94, 95). The core domain binds calcium ions, allowing them to interact with phospholipid membranes (95). The core

domain represents the large majority (80%) of the annexins protein, whereas the N-terminus likely confers specificity of action to each member of the annexin superfamily of proteins which includes annexins A1 and A2 (96). The function of binding with negatively charged phospholipids in a calcium-dependent manner is the characteristic feature of annexins (93, 94). An important feature of annexins is its capabilities to alter conformation upon binding to calcium cations. In the presence of calcium, annexins undergo a conformational restructuring which allows phospholipid binding, especially binding to acidic phospholipids (96). Calcium-binding motifs sustain the core region's interaction with phospholipids which is concomitant with N-terminal region conformational rearrangement to induce its amino acids exposure to the extracellular environment (96). These structural changes affect its ability to interact with potential receptors (96).

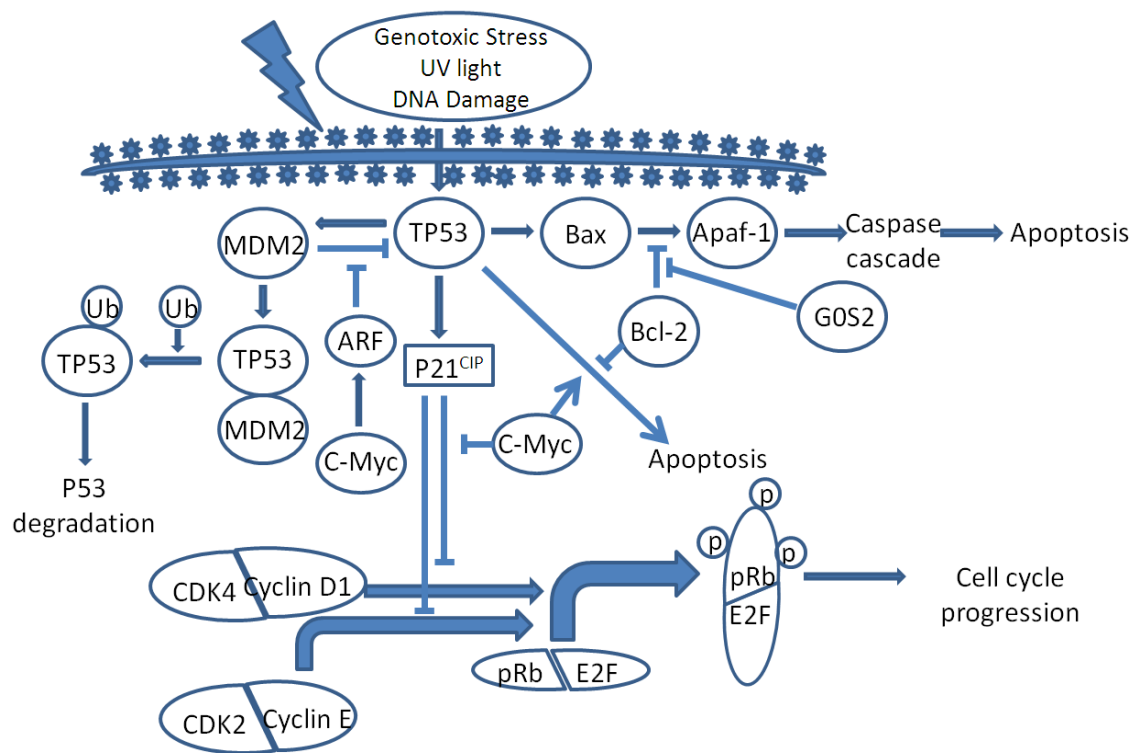
### **1.7 PAX2 and ovarian cancer**

*PAX* genes subgroups II and III, which are useful tumor markers, are frequently expressed in a wide variety of cancers, and their endogenous expression is required for the growth and survival of cancer cells (35). *PAX2* gene is frequently expressed in a panel of 406 common primary tumor tissues (97) and its expression is significantly higher in patients with metastatic disease as it is correlated with the proliferation index (98). *PAX2* promotes the cancer cell survival, motility and growth (35) and serves as a critical component of the multi-step oncogenic transformation process (65, 72, 99, 100). *PAX2* is a potential cancer therapy target as it suppresses cisplatin-induced apoptosis

and the silencing of PAX2 partially overcomes the resistance of renal cell carcinomas to chemotherapy *in vivo* (101). Little is known about the mechanism of PAX2-mediated protection from cell death (66). PAX2 which is highly expressed in serous papillary and clear-cell ovarian cancers is the best and most highly expressed discriminators of ovarian cancer (102). PAX2 protein plays an important role in tumor initiation and progression by transcriptional repressing the *TP53* gene promoter (99, 103, 104) (Figure 1-7). PAX2 interacts with Rb (105) and pRB can bind to the activation domain of PAX8 and activate PAX8 (106). Presently, the function and activation of PAX2 in ovarian cancer have not been clearly studied. This will be our future research focus. In this study, we further found that PAX2 is also overexpressed in other histological types of ovarian carcinomas – mucinous, clear cell and endometrioid ovarian carcinomas. Since mucinous, clear cell and endometrioid ovarian carcinomas are less responsive to the standard platinum/paclitaxel chemotherapy (26, 27), we explored whether PAX2 would be a potential therapeutic target for these ovarian carcinomas that express PAX2.

**Figure 1-7. TP53 signaling pathway.**

TP53 is expressed and undergoes post-translational modification upon DNA damage, which induces its accumulation in the nucleus. TP53 activation which is controlled through an autoregulatory loop involving Mdm2 turns on the transcription of p21<sup>CIP1</sup>. p21<sup>CIP1</sup> subsequently binds to and inhibits cyclin-dependent kinases which causes hypophosphorylation of retinoblastoma (Rb) and prevents the release of E2F and this blocks the G1-S transition. Deregulated expression of c-Myc, Bcl-2, or E2F can block some of the cellular effects of TP53. The binding of Mdm2 to TP53 induces TP53 for degradation and inhibits TP53-induced cell-cycle arrest and apoptosis (107-109). G0S2 encodes a mitochondrial protein that specifically interacts with Bcl-2 and prevents the formation of protective Bcl-2/Bax heterodimers which inhibits apoptosis (90).





## **CHAPTER 2**

### **MATERIALS AND METHODS**

## **2.1 Cell culture**

Twenty-eight human ovarian carcinoma cell lines RMUGL, TOV21G, TOV112D, MDAH-2774, IGROV1, ALST, OVCA432, PEO4, MCAS, OVCA429, OVCA433, ES2, SKOV3, RMG1, OVCA420, OVCAR3, RMUGS, MPSC1, HCH, KF, KK, KOC7C, OVAS, OWISE, OVSAYO, OVTOKO, ML38, ML46; and one immortalized ovarian surface epithelium cell line IOSE29 were obtained from The University of Texas MD Anderson Cancer Center, Houston, TX. All cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) containing 10% Fetal Bovine Serum and 1% penicillin/streptomycin in a humidified 5% CO<sub>2</sub> incubator at 37°C. We further silenced PAX2 expression in RMUGL, TOV21G, MDAH-2774 and IGROV1 ovarian carcinoma cell lines. The PAX2 silenced ovarian cancer cell lines, PLKO-puro and non-target control cell lines were cultured in RPMI containing 10% Fetal Bovine Serum and 1% penicillin/streptomycin with a 1 $\mu$ g/ml puromycin in a humidified 5% CO<sub>2</sub> incubator at 37°C.

## **2.2 Human subjects and tissue specimens**

Tissue specimens were obtained from the Department of Gynecologic Oncology Tumor Bank at The University of Texas MD Anderson Cancer Center and IRB approved. We used six human ovarian surface epithelia (HOSE), twenty-one type I ovarian cancer tissue samples and twenty-four type II ovarian serous carcinoma samples to perform Affymetrix microarrays gene expression profiling. We further screened PAX2 expression in different types of ovarian cancers which included 79 human ovarian

cancer tissue samples (16 high-grade serous ovarian cancer samples, 6 clear cell ovarian cancer samples, 8 low malignant potential/low-grade serous ovarian cancer samples, 27 mucinous ovarian cancer samples, 22 endometrioid ovarian cancer samples) and 9 human ovarian surface epithelium tissue samples using Affymetrix microarray analysis. Twenty-four clear cell ovarian cancer tissue sections, Fifty-one mucinous ovarian cancer tissue sections, and 263 high-grade serous ovarian carcinoma tissue sections were obtained from the University of Texas MD Anderson Cancer Center Pathology Department and used for PAX2 expression analysis using immunohistochemical analysis (IHC). The specialized gynecologic oncology pathologists in the University of Texas MD Anderson Cancer Center reviewed and graded all tissue samples according to the grading criteria outlined by Malpica et al (110).

### **2.3 Stable *PAX2* knockdown**

Twenty-eight parental human ovarian carcinoma cell lines were screened for PAX2 expression. Four cell lines (TOV21G, RMUGL, MDAH-2774, and IGROV1) had robust PAX2 expression. MISSION TRC shRNA Lentiviral Particles (Sigma-Aldrich SHVRS-NM\_000278, St. Louis, MO) targeting various regions of PAX2 (shRNA 15839, CCGGCGTCTCTTCCATCAACAGAATCTCGAGATTCTGTTGAT GGAAGAGACGTTTTT; shRNA 15840, CCGGCCCAAAGTGGTGGACAAGATTC TCGAGAATCTTGTCCACCACTTTGGGTTTTT; shRNA 15841, CCGGGATGAAG TCAAGTCGAGTCTACTCGAGTAGACTCGACTTGACTTCATCTTTTT) were used to transduce the ovarian cancer cell lines RMUGL, TOV21G, MDAH-2774 and

IGROV1, which had PAX2 expression. PLKO-puro Control (no insert sequence) (Sigma-Aldrich, St. Louis, MO) and shRNA Non-Target Control (insert sequence: CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTGTTTT) (Sigma-Aldrich, St. Louis, MO) were used as negative controls. We used multiplicity of infection (MOI) of 0.5, 1, 10 and 50 to transfect cells. The successful selecting MOI is 1. We next used 0.5 µg/ml, 0.8 µg/ml, 1 µg/ml and 5 µg/ml puromycin in RPMI containing 10% Fetal Bovine Serum and 1% penicillin/streptomycin to select the stable clones which was transfected with mission TRC shRNA lentiviral particles, PLKO-puro control and non-target control particles. We found 1 µg/ml is the best concentration for puromycin selection. The selected stable clones were routinely maintained in 10% Fetal Bovine Serum with 1% penicillin/streptomycin and 1 µg/ml puromycin in a humidified 5% CO<sub>2</sub> incubator at 37°C.

## **2.4 Affymetrix microarray analysis**

Tissue specimens were obtained from the Department of Gynecologic Oncology at The University of Texas MD Anderson Cancer Center and IRB approved. Frozen tissue samples containing more than 70% tumor cells were homogenized before RNA extraction. We performed RNA extraction according to Mini-prep RNeasy kit (Qiagen, Valencia, CA) per the manufacturer's protocol. Next, we generated cDNA using 5 µg of total RNA. We evaluated gene expression profiles using Affymetrix Human Genome U133 plus 2.0 (HG U133 plus 2.0) Gene Chips (Affymetrix, Santa Clara, CA) according to the Affymetrix Eukaryotic One-cycle protocol. cDNA was fragmented and

hydrolyzed at 94°C for 35 minutes in 24  $\mu$ L H<sub>2</sub>O and 6  $\mu$ L of 5 $\times$  fragmentation buffer (Affymetrix). Fragmented cDNA was hybridized with HG U133 plus 2.0 Gene Chip (Affymetrix) for 16 hours at 45°C in an Affymetrix Hybridization Oven 640 (Affymetrix). We then washed and stained arrays on Affymetrix Fluidics Station 450 (Affymetrix). Array data was scanned on Affymetrix GeneChip Scanner 3000 and fluorescence intensities were obtained using GeneChip Operating Software (Affymetrix). We processed raw images results (CEL files) using dChip software (111).

## **2.5 Antibodies and Taqman real-time RT-PCR primers**

The following antibodies were used for western blot analyses and immunohistochemical analysis. Rabbit polyclonal antibody to PAX2 Invitrogen immunodetection, 71-6000 (Zymed Laboratories, San Francisco, CA) which recognizes the expressed product of the PAX2 gene; mouse monoclonal anti-beta-actin antibody (Sigma-Aldrich Inc., St. Louis, MO); rabbit polyclonal PARP-1/2 (H-250) antibody sc-7150 (Santa Cruz Biotechnology, Santa Cruz, CA) which was raised against amino acids 764-1014 mapping at the C-terminus of PARP-1 of human origin; rabbit polyclonal anti-Annexin I antibody 71-3400 (Invitrogen, Camarillo, CA); rabbit polyclonal Anti-G0S2 antibody HPA010016 (Sigma-Aldrich, St. Louis, MO); goat anti-rabbit IgG-HRP sc-2004 (Santa Cruz Biotechnology); goat anti-mouse IgG-HRP sc-2005 (Santa Cruz Biotechnology). The following Taqman real-time RT-PCR primers were used in the real-time RT-PCR analysis and they were from Applied Biosystems, Carlsbad, CA. Taqman gene expression assay for PAX2 (assay ID number:

Hs00240858\_m1, HS01057417\_m1, HS01057423\_m1, HS01062572\_g1, HS01067916\_m1); Taqman gene expression assay for G0S2 (assay ID number: Hs00274783\_s1) (Applied Biosystems, Carlsbad, CA); and Taqman pre-developed assay reagent (human PPIA) which was used as endogenous control for results normalization.

## **2.6 Immunohistochemical analysis**

Paraffin tissue sections were obtained from The University of Texas MD Anderson Cancer Center Pathology Department and reviewed by specialized gynecologic oncology pathologists. We used the grading criteria outlined by Malpica et al (110). Briefly, immunohistochemical analysis was performed as follows (111). Deparaffinized specimens were immersed in 1× Reveal (Biocare Medical, Concord, CA) in a Coplin jar for antigen retrieval. Specimens were heated at 121°C for three minutes followed by one minute at 95°C in a Reveal decloaking chamber (Biocare Medical). Antibodies were placed onto the tissue sections with a 1:200 dilution at room temperature for four hours. Subsequently, tissue sections were probed with MACH 3 rabbit probe (Biocare Medical) and MACH 3 rabbit alkaline-phosphatase polymer (Biocare Medical). At last, we stained tissue sections using Vulcan Fast Red Chromagen Kit 2 (Biocare Medical) and counterstained them using CAT Hematoxylin (Biocare Medical). Nuclear staining and cytoplasmic staining of antibodies was analyzed. We took nuclear staining as the active staining for PAX2 because PAX2 encodes transcription factor which is mainly expressed in the nucleus when activated.

We graded the staining results according to the intensity and positive cell percentage as described (110).

## **2.7 WST-1 assay**

The cell proliferation reagent WST-1 (Roche Applied Science, Indianapolis, IN) was used to analyze cell viability. Cells were seeded at 8000 cells per well in 96-well plates. We then compared the cell viability of the parental ovarian cancer cell lines with PAX2 expression and PAX2 knockdown ovarian cancer cell lines at 1, 2, 4, 6, 7, 8 days. Before we measure cell proliferation for each date, 10  $\mu$ l of WST-1 reagent per well was added, and plates were incubated from 0.5 to 4 h in a humidified atmosphere (e.g., 37°C, 5% CO<sub>2</sub>). Plates were thoroughly shaken for 1 min, and then their light absorbance at 450 nm was measured against background controls using a microtiter plate reader.

## **2.8 Wound healing assay**

Parental ovarian cancer cells (RMUGL, TOV21G, MDAH-2774, and IGROV1) and PAX2 transfected ovarian cancer cells were cultured to confluence or near confluence (>90%) in a 6-well dish. Cells were subsequently rinsed with phosphate-buffered saline and starved overnight in low serum medium (1.5 ml; 0.5% - 0.1% serum in Dulbecco's modified Eagle's medium). On the day of the assay, a sterile 200  $\mu$ l pipette tip was used to scratch a cross-shaped wound through the cell lawn. Cells were rinsed with phosphate-buffered saline, and the low serum medium was replaced with

1.5 ml of medium containing 10% fetal bovine serum. After the wounds were created, the cultures were photographed using phase contrast at 10X magnification at 0, 5, 10, and 24 hours. The TScratch program (Computational Science & Engineering Laboratory, Zurich, Switzerland) was used to measure the open areas and analyze the data.

## **2.9 Nucleic acid extraction and cDNA synthesis**

Total RNA was isolated from ovarian cancer cell lines using the RNeasy Mini Kit (Qiagen, Valencia, CA). RNA quality and concentration were determined using Nanodrop, ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). One micro-gram of total RNA from each sample was used as template. cDNA synthesis was conducted using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). Briefly, each reaction contained 1 µg RNA, 10× RT buffer, 25× dNTPs, and 10× random hexamers in a 25 µL solution. The condition for cDNA amplification was 1 cycle at 25°C for 10 minutes, at 37 °C for 60 minutes, and at 95 °C for 5 minutes. One microliter of 50-mM EDTA was used to stop reactions and cDNA was diluted to 1:10 using sterile dH<sub>2</sub>O.

## **2.10 Taqman real-time RT-PCR**

RNA was extracted from ovarian cancer cell lines. We performed the reverse transcript reactions using the cDNA synthesized according to the method described above. Five microliter cDNA was combined with 7.5 µl 2x iQ supermix (Bio-Rad



Laboratories, Hercules, CA), 1.75  $\mu$ l dH<sub>2</sub>O, and 0.75  $\mu$ l 20x Taqman gene expression assay mix (PPIA or test genes) (Applied Biosystems, Carlsbad, CA). Each Taqman real-time RT-PCR was performed in triplicate. We normalized the results to cyclophilin A pre-developed Taqman expression gene assay (Applied Biosystems). mRNA quantification was determined using Bio-Rad CFX96 system (Bio-Rad Laboratories, Hercules, CA) with the condition of 1 cycle at 95°C for 3 minutes, followed by 40 cycles of 95°C for 10 seconds and 60°C for 30 seconds. All results were normalized using cyclophilin A. The mRNA copy number for tumor cell line samples was compared with that of reference RNA (Stratagene, Santa Clara, CA).

## **2.11 Western blot analysis**

Cells were cultured until 90% confluence and scraped in 2-3ml RPMI culture media. Cells were washed twice in 5ml PBS and centrifuged at 2000rpm for 2 minutes. We added 200 $\mu$ l cytoplasmic extraction buffer (Tris-Cl pH7.9 10mM, KCl 10mM, EDTA 0.1mM, EGTA 0.1mM) to cell pellet and suspended them slowly by pipetting. After 10 minutes of incubation on ice, 6.25 $\mu$ l 10% NP-40 was added into the cells. The tube was spun for 1 minute at 14000rpm and supernatant was the cytoplasmic protein extraction which would be transferred to a fresh 1.5ml tube. One hundred microliter of nuclear extraction buffer (Tris-Cl pH7.9 20mM, NaCl 400mM, EDTA 1mM, EGTA 1mM) was added to pellet which was incubated on ice for 45 minutes, while vortexed 10 seconds each 15 minutes. The tube was centrifuged at 12000rpm for 5 minutes at 4°C. The supernatant was the nuclear protein extraction. Protein concentration was

determined using a Beckman DU640B spectrophotometer (Beckman, Fullerton, CA). Fifty micro-gram of denatured protein was loaded and electrophoresed onto a 10% SDS-PAGE gel (Bio-Rad Laboratories, Hercules, CA), electroblotted on Hybond ECL nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK), and probed using different first antibodies according to the different target genes, such as rabbit polyclonal anti-PAX2 antibody (Zymed Laboratories, San Francisco, CA) at a 1:500 dilution; rabbit polyclonal anti-Annexin I antibody 71-3400 (Invitrogen, Camarillo, CA) at a 1:1000 dilution; rabbit polyclonal anti-G0S2 antibody HPA010016 (Sigma-Aldrich, MO, USA) at a 1:250 dilution; rabbit polyclonal PARP-1/2 (H-250) antibody sc-7150 (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:10,000 dilution; and mouse monoclonal anti-beta-actin antibody (Sigma-Aldrich Inc., St. Louis, MO) at a 1:10,000 dilution. The second antibodies, goat anti-mouse IgG- horseradish peroxidase sc-2005 (Santa Cruz Biotechnology) at a 1:10,000 dilution; and goat anti-rabbit IgG-horseradish peroxidase sc-2004 (Santa Cruz Biotechnology) at a 1:10,000 dilution were used according to the first antibodies. The bound antibodies were detected using an Amersham ECL Western blot detection reagent kit (GE Healthcare, Fairfield, CT). Nuclear expression of PAX2 was normalized with nuclear expression PARP-1/2. Total protein expression of Annexin I and G0S2 were normalized with beta-actin expression.

## **2.12 Cell cycle analysis**

We analyzed the cell cycle differences between PAX2 expressed ovarian cancer cell lines and PAX2 silenced ovarian cancer cell lines. Two million cells were fixed in

5ml of 95% EtOH for 1hour at room temperature. The fixed cells were stored at 4°C over night. We resuspended pellet and added 1 ml of 50µg/ml Propidium iodide (Santa Cruz Biotechnology, Santa Cruz, CA) and 100µl of 1mg/ml RNase (Sigma Chemicals, St. Louis, MO). The cells were incubated at 37°C for 30 minutes before cell cycle analysis. Cell cycle was analyzed by a Becton Dickinson FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA). The data were analyzed using the Becton Dickinson CellQuest Pro software package.

### **2.13 Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay (TUNEL assay)**

We used the APO-BRDU™ Kit (BD Bioscience, San Jose, CA) to detect the apoptosis of parental ovarian cancer cell lines with PAX2 expression and the PAX2 silenced ovarian cancer cell lines. In brief, one to two million cells were fixed in 70% (v/v) ethanol at -20°C over night. Fixed cells were stained by APO-BRDU™ Kit (BD Bioscience, Mountain View, CA). TUNEL assay was run on a Becton Dickinson FACSCalibur flow cytometer (BD biosciences, Mountain View, CA) which was equipped with a 488 nm Argon laser as the light source.

### **2.14 Side population analysis using Hoechst 33342 dye**

PAX2 expressed ovarian cancer cell lines and PAX2 silenced ovarian cancer cell lines were used to compare the side population percentage which represents the putative stem cells. The cells were detached by trypsinization, centrifuged and

resuspended in tissue culture medium containing 2% serum at a concentration of  $1 \times 10^6$  cells/mL. We used  $1 \times 10^7$  cells for analysis. The cells were labeled with 5.0  $\mu\text{g/mL}$  Hoechst 33342 dye (Molecular Probes-Invitrogen, Carlsbad, CA) at 37°C for 90 min either alone or in combination with ABCG2 efflux pump inhibitor Verapamil (100  $\mu\text{M}$ ) (Sigma-Aldrich). Cells were resuspended every 30 min during the incubation by inverting the tubes. At the end of the incubation, cells were centrifuged and resuspended in cold fresh tissue culture medium with 2% serum. The final concentration for cells is  $10^6$ - $10^7$  cells/mL. 7-Amino-actinomycin D (7AAD) was added to the cells to a final concentration of 2  $\mu\text{g/mL}$  prior to FACS analysis to exclude the dead cells from analysis. The SP analysis was done using a BD LSRII System (BD Biosciences). The Hoechst dye was excited with UV laser and its fluorescence was measured with both 675LP filter (Hoechst Red) and 440/40 filter (Hoechst Blue).

### **2.15 Reverse phase protein array (RPPA)**

Cell lysates were extracted by using lysis buffer (1% Triton X-100, 50mM HEPES, pH 7.4, 150mM NaCl, 1.5mM  $\text{MgCl}_2$ , 1mM EGTA, 100mM NaF, 10mM Na pyrophosphate, 1mM  $\text{Na}_3\text{VO}_4$ , 10% glycerol, containing freshly added protease and phosphatase inhibitors from Roche Applied Science Cat. # 04693116001 and 04906845001, respectively) and were serially diluted four times from undiluted to 1:16 dilution before they were arrayed on nitrocellulose-coated slides in a 11x11 format. Samples were probed with antibodies by a catalyzed signal amplification system and visualized by a diaminobenzadine colorimetric reaction. Slides were scanned on a

flatbed scanner to produce a 16-bit tiff image. Spots from the tiff images were identified, and their density was quantified using MicroVigene (VigeneTech Inc., Carlisle, MA). Relative protein levels for each sample were determined by interpolation of each dilution curve from the "standard curve" (supercurve) of the slide (antibody). All the data points were normalized for protein loading and transformed to linear values designated as "linear after normalization." The "linear after normalization" values were then transformed to natural log values and median-centered for hierarchical cluster analysis. Samples were probed with 217 antibodies. Based on our Qcsamples which was defined by the software, only 207 antibodies were included in the data analysis. A heat map was used to express overall patterns.

#### **2.16 Allophycocyanin-Annexin V staining (APC-Annexin V staining)**

One million cells were aliquoted into centrifuge tubes. Cells were centrifuged, and the supernatant was decanted. One hundred microliters of diluted (1:20 dilution) Annexin V (BD Pharmingen, Bedford, MA) were added to each sample, followed by incubation at room temperature in the dark for 15 min. Precipitates were washed with the Annexin V binding buffer and resuspended in 400  $\mu$ l binding buffer. Annexin V expression was determined using a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA), and single color samples were used to set compensation on the flow cytometer. Data were analyzed using the Becton Dickinson CellQuest Pro software package.

### **2.17 Animal studies**

Ten nude mice were obtained from the Department of Experimental Radiation Oncology at The University of Texas MD Anderson Cancer Center. Ovarian cancer cell lines TOV21G-non-target control and TOV21G-PAX2KD-shRNA15840 were injected to these mice, five per group. Cells were dissolved in 2:1 PBS/matrix gel mixture. One million cells in 100 $\mu$ l PBS/matrix gel mixture were subcutaneously injected into each mouse left flank. Tumor size was measured at every four days before mice were sacrificed. We sacrificed the mouse after the average tumor volume was more than 0.5 cm<sup>3</sup> in any one group of mice. Tumors were weighed after the mice were sacrificed. Student's t-test was used to compare the tumor weight difference between two groups.

## **CHAPTER 3**

### **THE EXPRESSION OF PAX2 IN OVARIAN CANCERS**

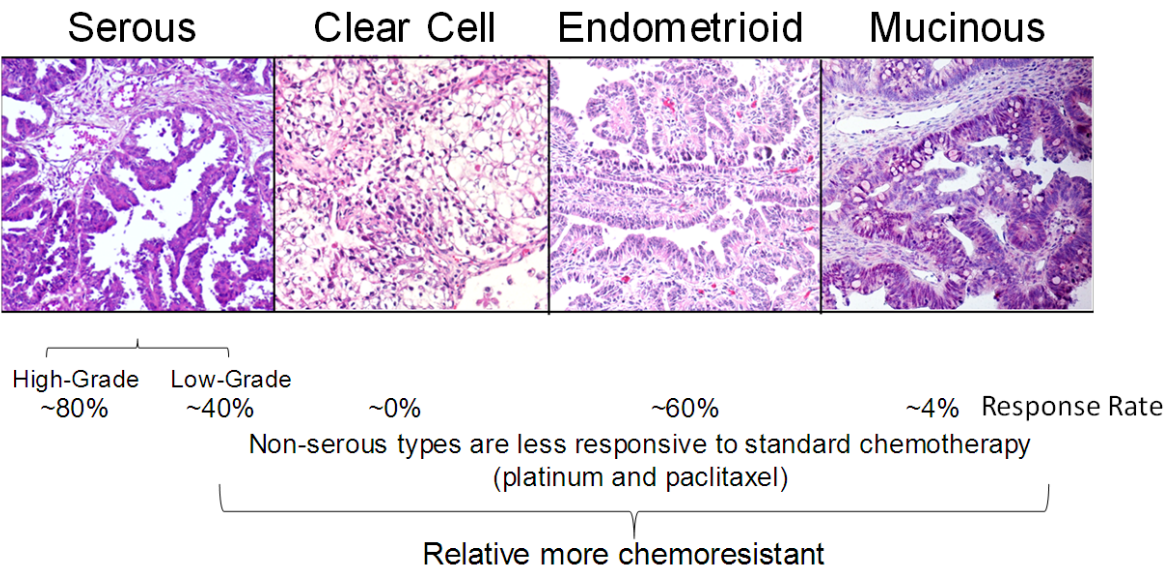
### **3.1 Introduction**

Ovarian cancer is the second most common gynecologic malignancy, and the most common cause of death among women who develop gynecologic cancers (5). Over 90% of ovarian cancers are epithelial neoplasms which are classified as serous (30-70%), endometrioid (10-20%), mucinous (5-20%), clear cell (3-10%), and undifferentiated (1%) ovarian cancers, with the five-year survival rates of 20-35%, 40-63%, 40-69%, 35-50%, and 11-29%, respectively. The rest are germ cell or stromal cell ovarian cancers (12, 13). Low-grade serous ovarian cancer was treated with platinum drugs (e.g. cisplatin and carboplatin) and taxanes (e.g. paclitaxel) and had a response rate of more than 80% (16). Non-serous ovarian cancers are relatively resistant to standard chemotherapy and need especially attention in the ovarian cancer treatment (Figure 3-1).



**Figure 3-1. Non-serous types of ovarian cancers are less responsive to standard chemotherapy.**

Non-serous types of ovarian cancers are less responsive to standard chemotherapy (platinum and paclitaxel). The response rate to chemotherapy is about 80% in high-grade serous ovarian cancer, 40% in low-grade serous ovarian cancer. As to clear cell ovarian cancer, endometrioid ovarian cancer, mucinous ovarian cancer, the response rates are 0%, 60% and 4% respectively.



*PAX2* is one of the nine *PAX* genes which have a conserved DNA sequence motif called the paired box, a 128 amino acid domain in the amino-terminal portion of the protein (40, 73). *PAX2* regulates tissue development and cellular differentiation in embryos (44, 112-124). *PAX2* also promotes cell proliferation, oncogenic transformation, cell-lineage specification, migration, and survival (97, 125). Unattenuated *PAX2* has been found in several cancer types (126) and immunotherapy targeting *PAX2* is a promising method to treat ovarian cancer patients (24). Thus, deciphering downstream targets and functions of *PAX2* in the development and progression of ovarian cancer will facilitate ovarian cancer immunotherapy.

Our former research (111) showed for the first time that *PAX2* had a high expression in low malignant potential and low-grade ovarian serous carcinomas and a low expression in high-grade serous carcinomas (Figure 3-2). This enhanced the current conception about ovarian cancer development, that is, low grade and high grade ovarian cancer developed from different pathways with low-malignant potential tumors potentially developing along a disease continuum to low-grade cancers. Our present research further verified that *PAX2* had a higher expression in the other histological types of ovarian cancers including mucinous ovarian cancer, endometrioid ovarian cancer and clear cell ovarian cancer. We also evaluated the mechanism of *PAX2* function in ovarian carcinomas in this dissertation. This will provide the theoretical basis for individualized ovarian cancer treatment by targeting *PAX2*.

**Figure 3-2. PAX2 is highly expressed in LMP and low-grade serous ovarian cancer.**

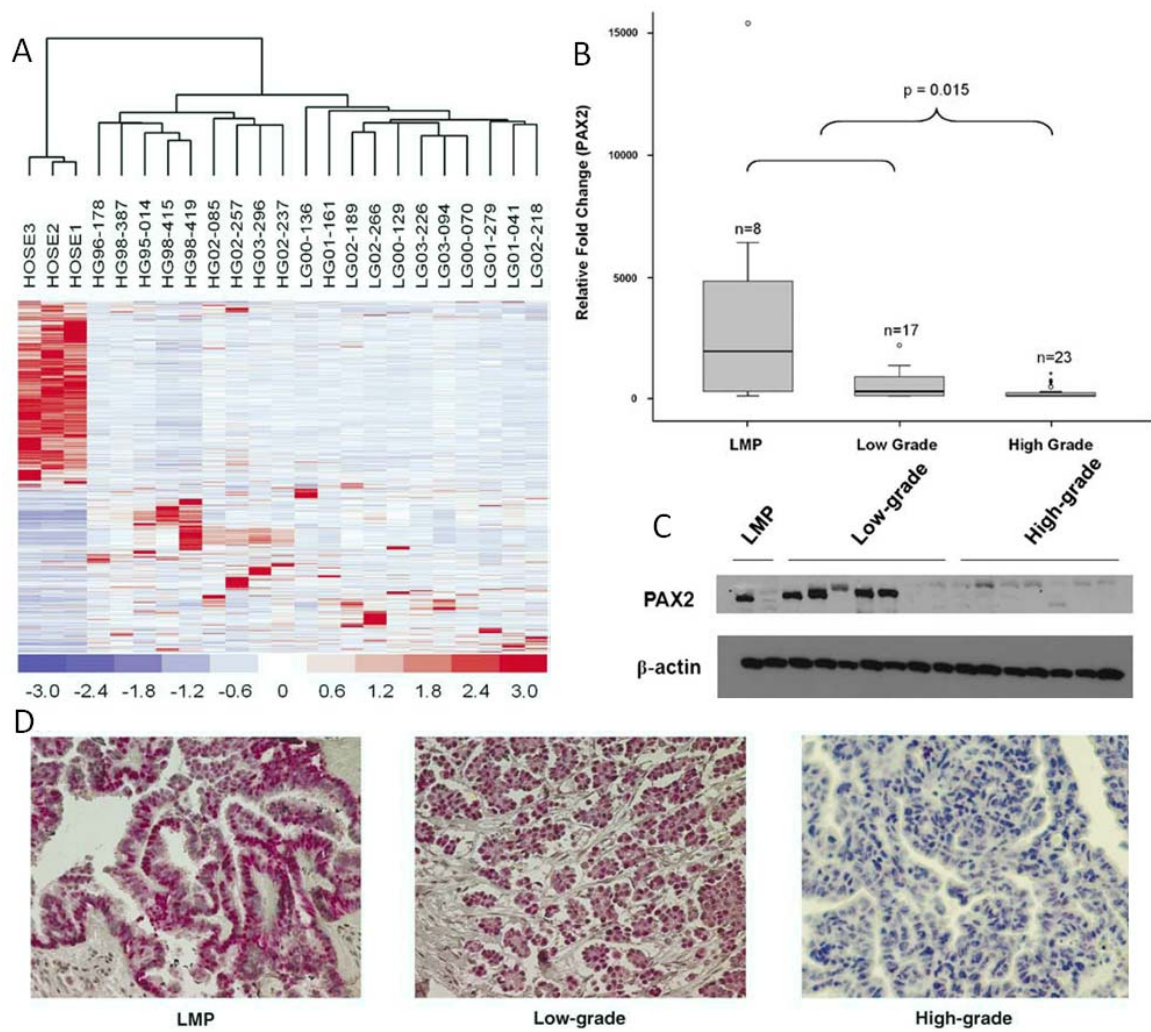
*Reprinted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: (Celestine S Tung, Samuel C Mok, Yvonne T M Tsang, Zhifei Zu, Huijuan Song, Jinsong Liu, Michael T Deavers, Anais Malpica, Judith K Wolf, Karen H Lu, David M Gershenson, Kwong-Kwok Wong. Mod Pathol. 2009 Sep;22(9):1243-50.), copyright (2009)*

(A) Affymetrix Microarray data for PAX2 expression. Gene expression cluster analysis of RNA extracted from samples of three normal human ovarian surface epithelia, ten low-grade, and ten high-grade ovarian serous carcinomas.

(B) PAX2 mRNA expression in ovarian cancers. Comparison of PAX2 mRNA expression between eight low malignant potential ovarian cancers, seventeen low-grade and twenty-three high-grade ovarian carcinoma samples. The box is bounded by the 25<sup>th</sup> and 75<sup>th</sup> percentile with the median expression level depicted by the line in the box. Outlying values are drawn individually. Expression of PAX2 in high-grade is significantly lower than either low malignant potential or low-grade tumors (P=0.015).

(C) Western blot examination of PAX2 protein expression in ovarian cancer patient samples.

(D) PAX2 expression in ovarian cancer patient samples using immunohistochemical staining. Examples of PAX2 immunohistochemical staining of individual paraffin sections from low malignant potential tumors, low-grade and high-grade ovarian serous carcinomas (×200 magnification).



### **3.2 Aims**

According to our former researches, PAX2 is highly expressed in low-grade serous ovarian cancers and LMP ovarian cancers, while it has a lower expression in high-grade serous ovarian cancers. PAX2's expression level in the other types of ovarian cancers is one question that we need to further explain. Our aim is to clarify or characterize PAX2 expression in the other histological types of ovarian cancers. We used Affymetrix microarray analysis, immunohistochemical analysis to validate PAX2's expression in the other types of ovarian cancer patient samples.

### **3.3 Results and Discussion**

#### **3.3.1 High *PAX2* expression in non-serous ovarian cancers and low *PAX2* expression in high grade serous ovarian cancers**

*PAX2* is one of nine *PAX* genes which regulate tissue development and cellular differentiation in embryos by promoting cell proliferation, cell-lineage specification, migration and survival (35, 36). Our former research (111) found that *PAX2* is one of the most upregulated genes in low-grade ovarian serous carcinoma (Figure 3-2A) by gene expression profiling. We (111) also validated this result by real-time RT-PCR (Figure 3-2B), western blot (Figure 3-2C) and immunohistochemical analyses (Figure 3-2D).

Our gene expression profiling data further confirmed this observation (Figure 3-3). We initially evaluated gene expression profiles on twenty-one LMP and low-grade serous carcinoma samples, twenty-four high-grade serous carcinoma samples and six normal ovarian surface epithelia (HOSE) samples from The University of Texas MD Anderson Cancer Center using Affymetrix microarray analysis. We found eight highly overexpressed genes and fourteen significantly underexpressed genes in LMP and low-grade serous ovarian cancer compared to high-grade serous ovarian cancer. *PAX2* is one of the overexpressed genes with a fold increase of 4.78 ( $p=0.04$ ). Subsequently, we evaluated *PAX2* expression profiles using Affymetrix microarray analysis on different types of ovarian cancer patient samples (eight LMP and low-grade serous carcinoma samples, sixteen high-grade serous carcinoma samples, six clear cell ovarian cancer samples, twenty-seven mucinous ovarian cancer samples, twenty-two endometrioid ovarian cancer samples and nine normal ovarian surface epithelia (HOSE) samples (Figure 3-3B). We found that *PAX2* was highly expressed in non-serous ovarian tumors (clear cell, endometrioid cell, mucinous cell ovarian cancers) which are more resistant to the standard chemotherapy using platinum drugs (e.g. cisplatin and carboplatin) and taxanes (e.g. paclitaxel). To verify *PAX2* expression in different types of ovarian cancer samples, we did the immunohistochemical analysis by using twenty-four clear cell ovarian cancer tissue paraffin sections and fifty-one mucinous ovarian cancer tissue paraffin sections. Tumor sections were either lacked nuclear staining or had robust nuclear staining. No weak or moderate strength staining was observed. The number of *PAX2* positive tumor cells varied from 1-100%. In twenty-four clear cell ovarian cancer

tissue sections, 20.8%, 41.7% and 20.8% were 1-25%, 25-50%, and 75-100% positive respectively. In 51 mucinous ovarian cancer tissue sections, 24.3%, 28.6% and 20% were 1-25%, 25-50%, and 75-100% positive respectively. In our former research about 263 high-grade serous ovarian carcinoma tissue paraffin sections, 2%, 5% and 3% were  $\leq 1\%$ , 10-50%, and 75-100% positive respectively. In conclusion, non-serous ovarian cancer tissue sections showed robust nuclear staining of PAX2. High-grade serous ovarian cancer tissue sections showed no nuclear staining or extremely faint cytoplasmic staining of PAX2 (Figure 3-3C). This is consistent with, and further supported our former data about PAX2 expression in ovarian cancers (111). According to our statistical analysis, non-serous ovarian cancer sections had significantly higher robust nuclear PAX2 staining than high-grade serous ovarian cancer sections ( $p < 0.001$ ) (Table 3-1). *PAX2* may therefore have value as a potential therapeutic target for non-serous ovarian cancers.

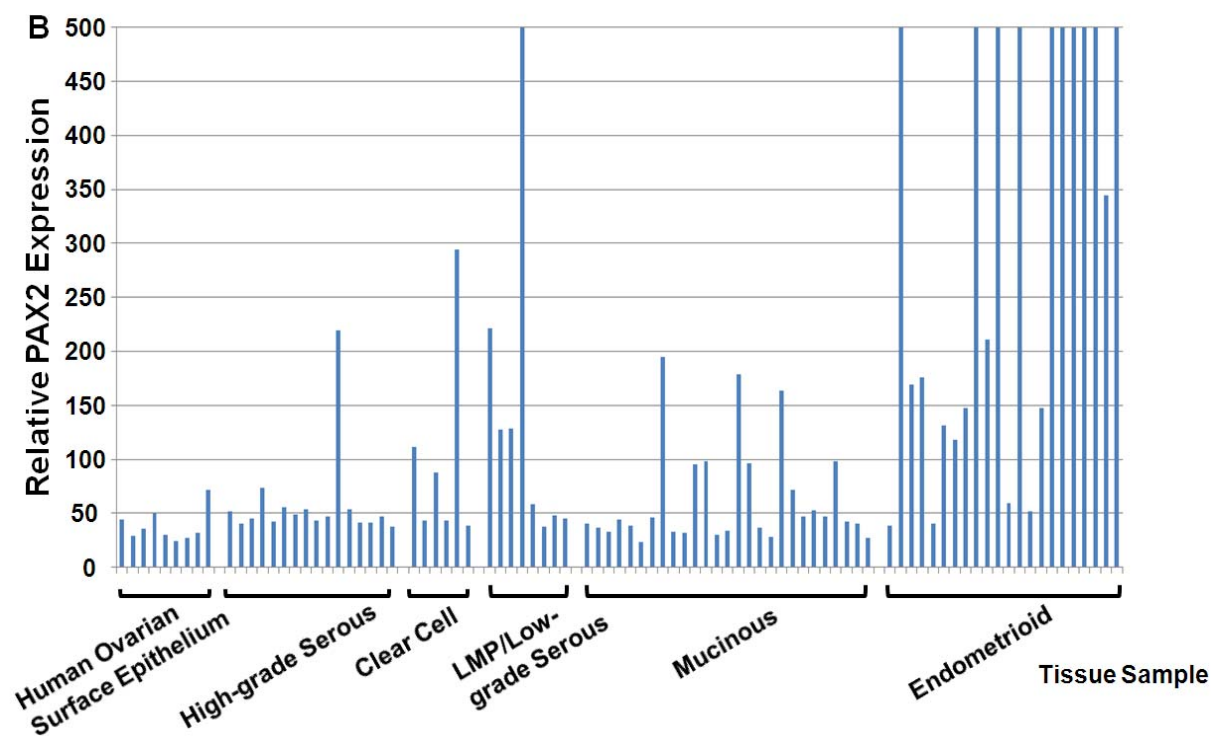
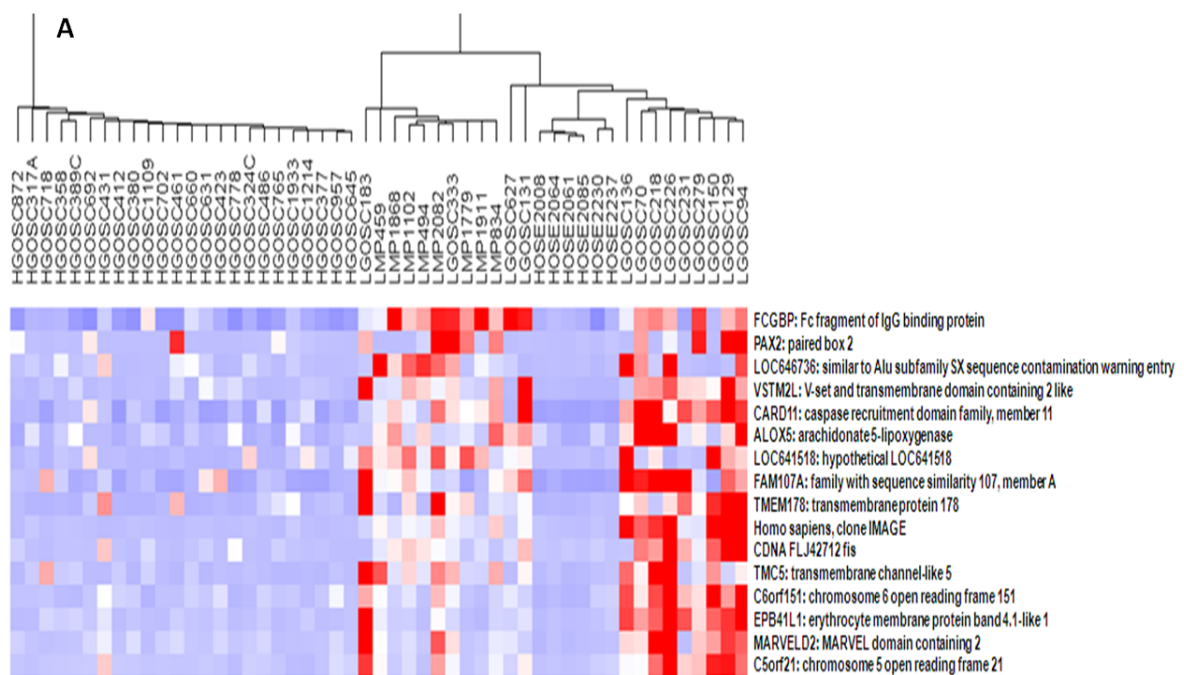
**Figure 3-3. PAX2 expression in different types of ovarian cancers.**

(A) Affymetrix Microarray data for *PAX2* expression. Gene expression cluster analysis of RNA extracted from normal human ovarian surface epithelia (HOSE), LMP, low-grade serous carcinoma and high-grade serous carcinoma. *PAX2* is one of eight highly overexpressed genes in low-grade serous ovarian cancer compared to high-grade serous ovarian cancer. ( $p=0.04$ )

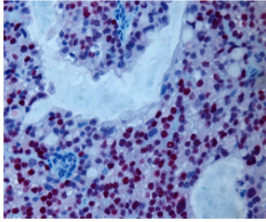
(B) Microarray analysis revealed *PAX2* was highly expressed in non-serous ovarian cancers compared with high-grade serous ovarian cancers. *PAX2* expression was examined in 9 human ovarian surface epithelium samples and 79 human ovarian cancer clinical samples that included all ovarian cancer histological subtypes (16 high-grade serous ovarian cancer samples, 6 clear cell ovarian cancer samples, 8 low malignant potential (LMP)/low-grade serous ovarian cancer samples, 27 mucinous ovarian cancer samples, and 22 endometrioid ovarian cancer samples).

(C) *PAX2* immunohistochemical staining in ovarian cancer tissue sections. Examples of *PAX2* immunohistochemical staining of paraffin sections from clear cell ovarian cancer, mucinous cell ovarian cancer, endometrioid cell ovarian cancer and high-grade serous cell ovarian cancer patient tissue samples (200× magnification).

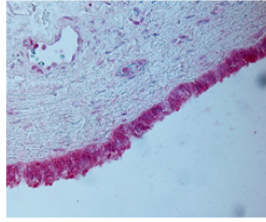




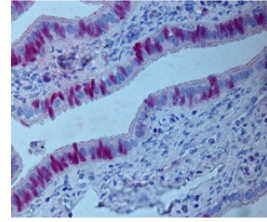
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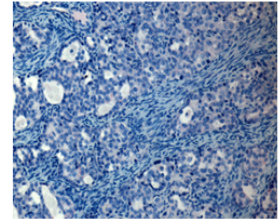
**Clear Cell  
Ovarian Cancer**



**Mucinous Cell  
Ovarian Cancer**



**Endometrioid Cell  
Ovarian Cancer**



**High-grade Serous  
Cell Ovarian Cancer**

**Table 3-1. PAX2 immunohistochemical nuclear staining statistic analysis.**

Sample	Number of samples with nuclear staining (%)	p-value(compared to high-grade)
Clear cell carcinoma (n=24)	20 (83.3%)	<0.001
Mucinous ovarian cancer (n=70)	51 (72.9%)	<0.001
High-grade (n=263)	27 (10.3%)	-----

## **CHAPTER 4**

### **SILENCING PAX2 EXPRESSION IN OVARIAN CANCER CELL LINES USING LENTIVIRAL TRANSDUCTION SYSTEM**

## **4.1 Introduction**

As our former immunohistochemical results had shown, PAX2 had a high expression in non-serous ovarian cancer, which includes mucinous, endometrioid, and clear cell carcinomas, while PAX2 expression was low in high-grade serous ovarian cancer. In this chapter, we further validated PAX2 expression at the molecular level by using different ovarian cancer cell lines. We silenced PAX2 expression using MISSION TRC shRNA Lentiviral Particles transduction system which is relatively reliable and consistent as they target genes at the nuclear DNA instead of mRNA processing process. We are the first one to explore the function of PAX2 in ovarian cancer development and progression.

## **4.2 Aims**

PAX2 is highly expressed in non-serous ovarian cancer patient samples and these patients are relatively chemo resistant compared with serous ovarian cancer patients. We hypothesize that PAX2 maybe a potential therapeutic target for non-serous ovarian cancer patients. Our aim is to identify the role of PAX2 in ovarian cancer progression. To validate PAX2's function, we identified the PAX2 positive non-serous ovarian cancer cell lines and knocked down their PAX2 expression. Thus, we used Taqman real-time RT-PCR, western blot to screen PAX2 expression in ovarian cancer cell lines. MISSION TRC shRNA Lentiviral transduction system was further used to

silence PAX2 expression in these PAX2 positive ovarian cancer cell lines. Western blot analysis and Taqman real-time RT-PCR were used to confirm PAX2 silencing results.

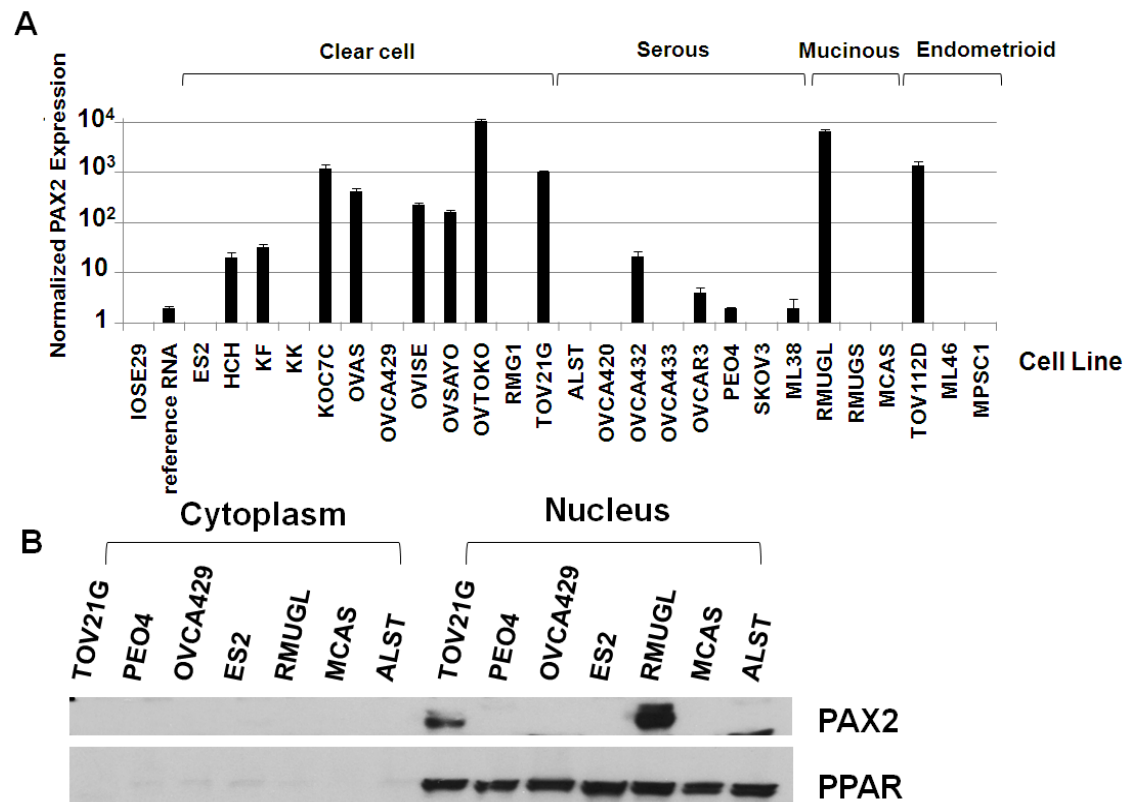
## **4.3 Results and Discussion**

### **4.3.1 Screening ovarian cancer cell lines for PAX2 expression**

We screened twenty-eight human ovarian carcinoma cell lines (eight serous ovarian cancer cell lines, twelve clear cell ovarian cancer cell lines, three mucinous ovarian cancer cell lines, five endometrioid ovarian cancer cell lines) and one immortalized ovarian surface epithelium cell line IOSE29 for PAX2 nuclear expression by using western blot analysis. Figure 4-1A shows part of ovarian cancer cell lines' PAX2 expression using Taqman real-time RT-PCR. We also confirmed our results using western blot analysis at the mRNA level (Figure 4-1B). We found that twelve of the cell lines, OVCAR3, OVCA432, TOV21G, TOV112D, RMUGL, HCH, KF, KOC7C, OVAS, OWISE, OVSAYO, and OVTOKO, had detectable higher PAX2 expression from 2 to 3220 folds comparing to the reference RNA (Figure 4-1). These PAX2 positive cell lines are mainly non-serous ovarian cancer cell lines, such as mucinous ovarian cancer cell line RMUGL, clear cell ovarian cancer cell line TOV21G, endometrioid ovarian cancer cell line MDAH-2774 and IGROV1. This is consistent with the human samples as to the PAX2 expression. We will further use these cell lines which express PAX2 to study PAX2's function.

**Figure 4-1. PAX2 is over-expressed in several types of ovarian cancer cell lines.**

(A) Screening of 26 ovarian cancer cell lines using Taqman real-time RT-PCR showed that PAX2 was overexpressed in clear cell, serous, mucinous, and endometrioid ovarian cancer cell lines. (B) Examples of PAX2 expression in different ovarian cancer cell lines. Western blot analysis verified the PAX2 expression level in 7 ovarian cancer cell lines.



#### **4.3.2 Silencing PAX2 expression in PAX2 positive ovarian cancer cell lines**

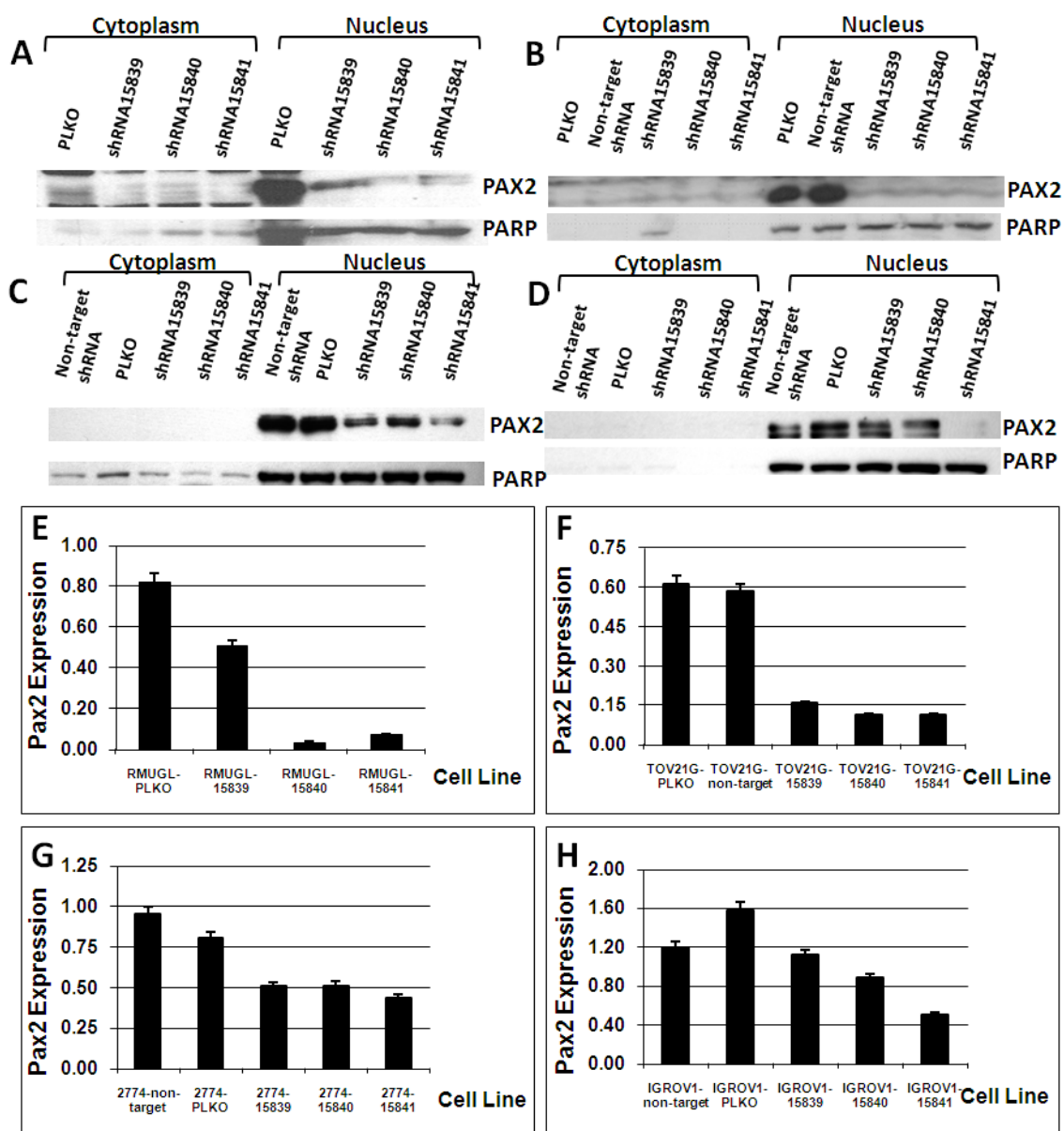
We found that fourteen of 28 cell lines, OVCAR3, OVCA432, TOV21G, TOV112D, RMUGL, HCH, KF, KOC7C, OVAS, OVISe, OVSAYO, MDAH-2774, IGROV1 and OVTOKO, had detectable PAX2 expression from 2 to 3220 fold higher as compared to reference total RNA which is extracted from 10 human cell lines derived from different tissues providing broad gene coverage (Figure 4-1A). PAX2 expression was particularly high in TOV21G, RMUGL, MDAH-2774 and IGROV1 ovarian cancer cell lines. These four cell lines were used for our PAX2 silencing experiments and future functional study about PAX2. We used MISSION TRC shRNA Lentiviral Particles to transfect the ovarian cancer cell lines, TOV21G, RMUGL, MDAH-2774 and IGROV1. After stable silencing PAX2 expression, we used western blot assay to verify efficiency of knocking down at the protein level as shown in Figure 4-2. Compared with the PLKO control in the RMUGL cell line, PAX2 expression was downregulated by 37.7%, 95.8%, and 91.1% in shRNA 15839-, shRNA 15840-, and shRNA 15841-treated cells, respectively (Figure 4-2A, E). It was also downregulated by 73.3%, 81.0%, and 80.9% in shRNA 15839-, shRNA 15840-, and shRNA 15841-treated cells when compared with the non-target control in the TOV21G cell line (Figure 4-2B, F). Compared with the non-target control, a knockdown of 46.5%, 46.3%, and 54.2% in shRNA 15839-, shRNA 15840- and shRNA 15841-treated cells was obtained for the MDAH-2774 cell line (Figure 4-2C, G). Downregulation of PAX2 in the IGROV1 cell line was 6.4%, 26.1%, and 58.3% in shRNA 15839-, shRNA 15840-, and shRNA 15841-treated cells compared with the non-target control (Figure 4-2D, H). We



normalized PAX2 nuclear expression with PARP-1/2 nuclear expression. PAX2 downregulation was also confirmed by Taqman real-time RT-PCR (Figure 4-3). The knockdown effect was especially robust using shRNA 15841. RMUGL and TOV21G had a larger response to shRNA than MDAH-2774 and IGROV1. In summary, our PAX2 Mission shRNA lentiviral particle transduction system was efficient and effective. We can use this system to do the future functional study of PAX2.

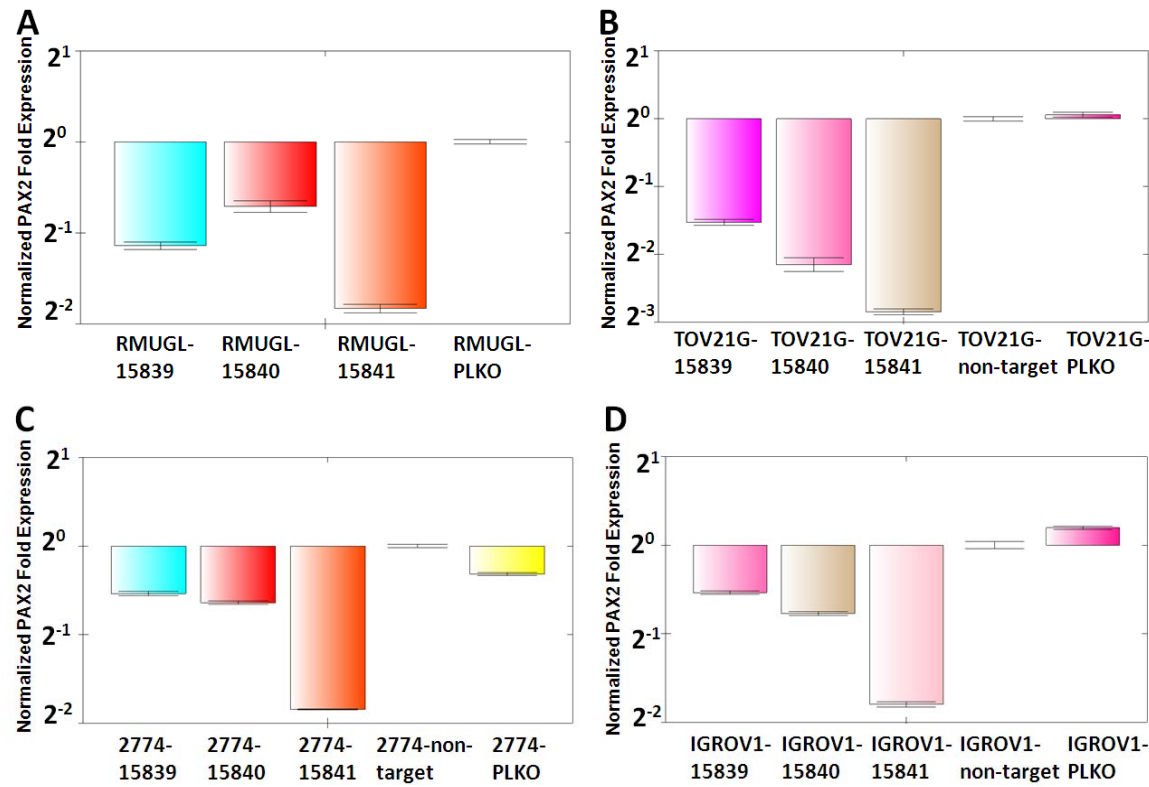
**Figure 4-2. Silencing PAX2 expression in different ovarian cancer cell lines using MISSION TRC shRNA Lentiviral Particles.**

Western blot analysis was used to examine PAX2 expression. PAX2 expression was normalized on the basis of the western blot results. PLKO and non-target shRNA were the negative controls. (A, E) Mucinous ovarian cancer cell line RMUGL. (B, F) Clear cell ovarian cancer cell line TOV21G. (C, G) Endometrioid ovarian cancer cell line MDAH-2774. (D, H) Endometrioid ovarian cancer cell line IGROV1. shRNAs 15839, 15840, and 15841 were PAX2-targeted shRNAs, which knocked down PAX2 expression in the ovarian cancer cell lines to various degrees.



**Figure 4-3. Taqman real-time RT-PCR validation of *PAX2* knockdown in ovarian cancer cell lines.**

Taqman real-time RT-PCR was used to check *PAX2* expression in different ovarian cancer cell lines after silencing *PAX2* expression. All results were normalized using cyclophilin A. (A) Mucinous ovarian cancer cell line RMUGL. (B) Clear cell ovarian cancer cell line TOV21G. (C) Endometrioid ovarian cancer cell line MDAH-2774. (D) Endometrioid ovarian cancer cell line IGROV1. Compared with the control, silencing *PAX2* expression in these ovarian cancer cell lines was successful.



## **CHAPTER 5**

# **DOWNREGULATION OF PAX2 SUPPRESSES OVARIAN CANCER CELL GROWTH**

## 5.1 Introduction

Two aspects will affect the cell growth: cell viability and cell proliferation. *PAX2* promotes cell growth in prostate epithelial and prostate cancer cell lines (127). We hypothesize that *PAX2* will have similar effects in non-serous ovarian cancer development and progression. After silencing *PAX2* expression in *PAX2* positive ovarian cancer cell lines, WST1 assay was used to measure the cell viability. Flow cytometry TUNEL assay was used to measure changes in the cell cycle phases which reflected the proliferation rate.

In mucinous cell ovarian cancer cell line RMUGL, we compared the cell viability in *PAX2* silenced cell lines and PLKO control *PAX2* positive cell lines at days 1, 2, 4, 6, 8 which reflected the metabolic state of the cells. In clear cell ovarian cancer cell line TOV21G, we compared the cell viability in *PAX2* silenced cell lines, PLKO control and non-target control *PAX2* positive cell lines at days 1, 2, 4, 6, 8. In endometrioid cell ovarian cancer cell line MDAH-2774 and IGROV1, we compared the cell viability in *PAX2* silenced cell lines, PLKO control and non-target control *PAX2* positive cell lines at days 1, 2, 4, 6, 7. Subsequently, flow cytometry was used to check the changes in the cell cycle phases after silencing *PAX2* expression in *PAX2* positive ovarian cancer cell lines. The cells were subjected to propidium iodide incorporation and flow cytometry was used to measure differences in cell cycle phases.

*PAX2* has been shown to increase cell motility in kidney cancer cell lines (127). We examined its effect on ovarian cancer cell motility by using *PAX2* silencing system and examined cell motility by using wound healing assay. We compared the cell

motility in *PAX2* silenced mucinous ovarian cancer cell lines RMUGL and PLKO control *PAX2* positive cell lines at 0h, 5h, 10h, and 24h respectively. As to the clear cell ovarian cancer cell line TOV21G, endometrioid ovarian cancer cell lines MDAH-2774 and IGROV1, we compared cell motility in *PAX2* silenced cell lines, PLKO control and non-target control *PAX2* positive cell lines at 0h, 5h, 10h, and 24h respectively. Subsequently, TScratch program (Computational Science & Engineering Laboratory, Switzerland) was used to quantitatively analyze wound healing assay results.

## **5.2 Aims**

The aim is to study the effect of *PAX2* downregulation on ovarian cancer growth *in vitro* and *in vivo*. We used WST1 assay and wound healing assay to measure cell viability and cell motility respectively following *PAX2* downregulation. Mouse xenograft model was used to study the effect of downregulation *PAX2* on tumor growth.

## **5.3 Results and Discussion**

### **5.3.1 *PAX2* knockdown decreased cell viability**

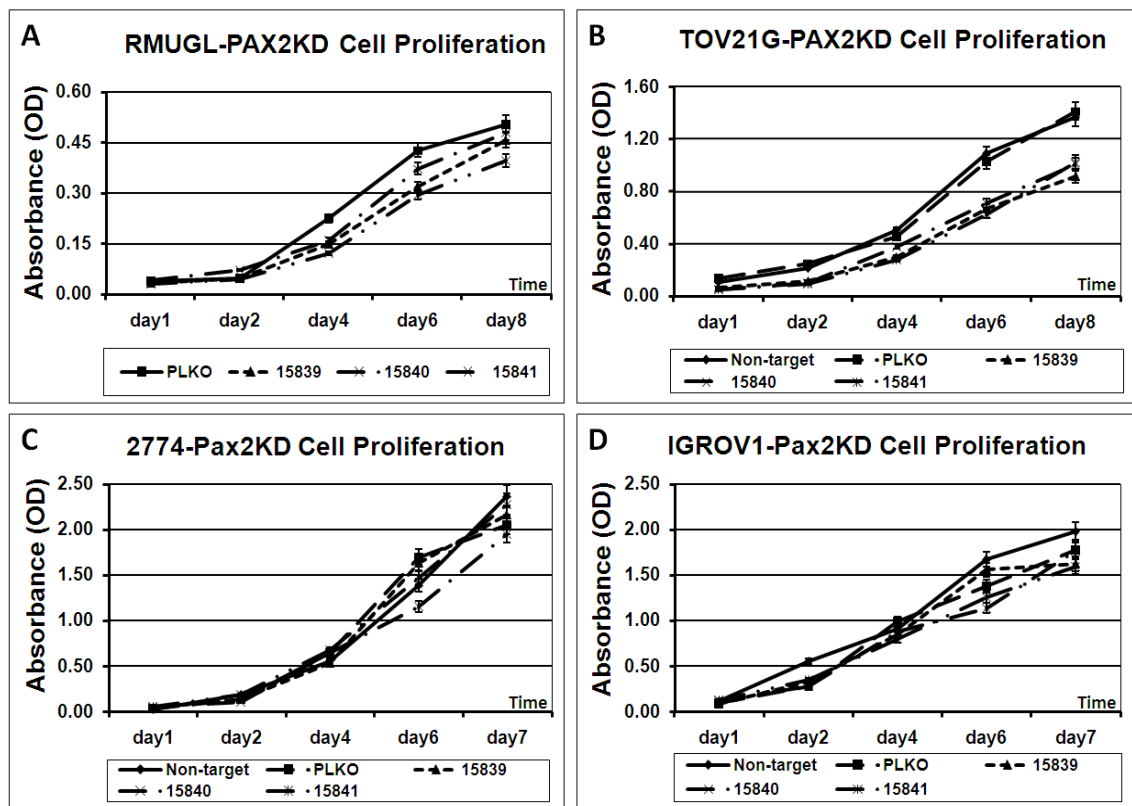
After successfully silencing *PAX2* expression in ovarian cancer cell lines, RMUGL, TOV21G, MDAH-2774 and IGROV1, we did WST1 assay on both the original ovarian cancer cell lines and *PAX2* silenced cell lines. As shown in Figure 5-

1A, compared with the PLKO control cell line, all the PAX2 silenced RMUGL cell lines had a decreased proliferation rate. The same was true for clear cell ovarian cancer cell line TOV21G (Figure 5-1B) and endometrioid ovarian cancer cell lines MDAH-2774 (Figure 5-1C) and IGROV1 (Figure 5-1D). PAX2 silenced cell lines had a lower proliferation rate compared with the PAX2 positive control cell lines ( $P < 0.001$ ). As WST1 assay measured cell viability which represented the cell metabolism rate, the PAX2 silenced cell lines had a decreased metabolism rate compared with the PAX2 positive control cell line which indicates that PAX2 positive ovarian cancer cell lines had a higher metabolism rate. The higher metabolism rate indicates that the cells are more active and those cells are more likely to have a higher proliferation rate. The consequence of high proliferation rate is a rapid cell growth rate. In brief, we concluded that silencing PAX2 expression decreased cell growth in ovarian cancer cell lines. This is consistent with the studies of Bose et al (127) which showed that angiotensin II upregulated PAX2 expression in prostate epithelial cells resulted in increased cell growth. Thus, we concluded that downregulating PAX2 expression decreased ovarian cancer cell growth by decreasing cell viability.



**Figure 5-1. Cell viability analyses confirmed the effects of PAX2 knockdown in ovarian cancer cell lines.**

The WST-1 assay was used to compare cell viability among the PAX2 knockdown and PAX2-expressing ovarian cancer cell lines. PLKO and non-target shRNA were the negative controls. (A) Mucinous ovarian cancer cell line RMUGL. (B) Clear cell ovarian cancer cell line TOV21G. (C) Endometrioid ovarian cancer cell line MDAH-2774. (D) Endometrioid ovarian cancer cell line IGROV1. shRNAs 15839, 15840, and 15841 were PAX2-targeted shRNAs, which knocked down PAX2 expression and decreased cell viability to various degrees.



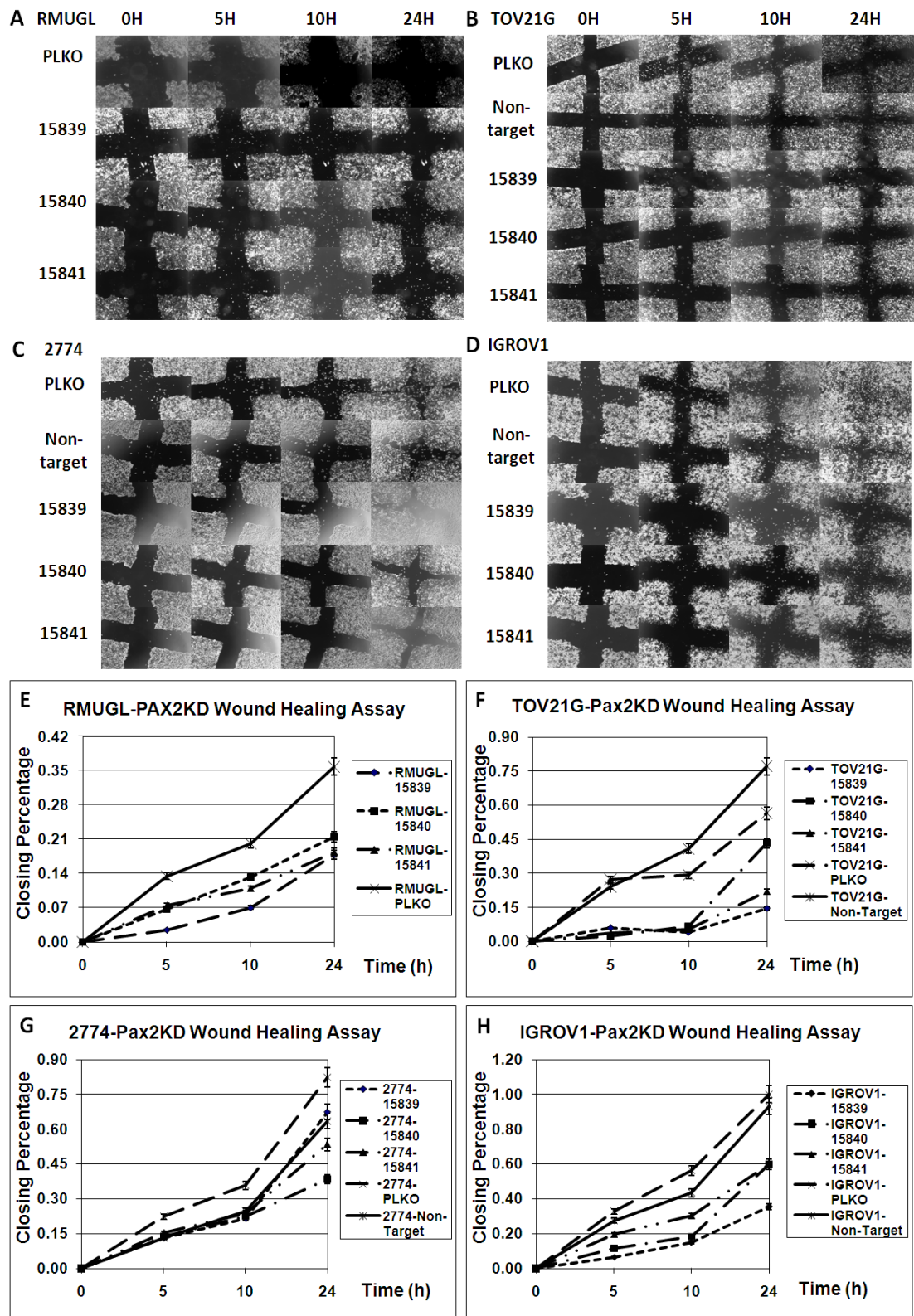
### **5.3.2 PAX2 knockdown decreased cell motility**

We performed a wound healing assay to analyze the effects of silencing PAX2 expression in ovarian cancer cell lines. We used ovarian cancer cell lines, RMUGL, TOV21G, MDAH-2774 and IGROV1. After stable silencing PAX2 expression in these cell lines, the cultures were photographed at 0h, 5h, 10h and 24h respectively. We used TScratch program to measure the open areas. We found that compared with control cell lines which had PAX2 expression, PAX2 silenced ovarian cancer cell lines had bigger open areas and lower cell motility as shown in figure 5-2. In mucinous ovarian cancer cell line RMUGL, PLKO control cell line had a stable increase of closing area from 0 hour to 24 hours, while the PAX2 silenced cell lines RMUGL-15839 and RMUGL-15840 had a stable closing rate of 0.01% from 0 hour to 24 hours which indicated that they almost did not move in this periods of time. RMUGL-15841 PAX2 silenced cell line began a slow movement at 5 hours and the closing rate is stable and consistent at a lower rate comparing to the RMUGL-PLKO control cell line ( $P<0.001$ ) (Figure 5-2A, Figure 5-2E). As to the clear cell ovarian cancer cell line TOV21G, TOV21G-non-target control cell line had the highest closing rate, followed by TOV21G-PLKO control cell line. TOV21G-15839, TOV21G-15840 and TOV21G-15841 PAX2 silenced cell lines had a stable slow closing rate from 0 hour to 10 hours which was much lower than PLKO and non-target control cell lines ( $P<0.001$ ) (Figure 5-2B, Figure 5-2F). As to the endometrioid cell ovarian cancer cell lines MDAH-2774 and IGROV1, PLKO control cell lines had the highest closing rate, followed by non-target control cell lines. 15839, 15840 and 15841 PAX2 silencing cell lines had a stable slow closing rate from 0 hour

to 10 hours which was much lower than PLKO and non-target control cell lines ( $P<0.001$ ) (Figure 5-2C, Figure 5-2D, Figure 5-2G, Figure 5-2H). These results confirmed our hypothesis that downregulating PAX2 expression decreased cell motility, and suggest that PAX2 is a potential oncogene and can be the therapeutic target for non-serous ovarian cancer patients.

**Figure 5-2. Cell motility analyses confirmed the effects of PAX2 knockdown in ovarian cancer cell lines.**

Wound healing assay in PAX2 knockdown and PAX2-expressing ovarian cancer cell lines; PLKO and non-target shRNA were the negative controls. (A, E) Mucinous ovarian cancer cell line RMUGL. (B, F) Clear cell ovarian cancer cell line TOV21G. (C, G) Endometrioid ovarian cancer cell line MDAH-2774. (D, H) Endometrioid ovarian cancer cell line IGROV1. shRNAs 15839, 15840, and 15841 were PAX2-targeted shRNAs, which knocked down PAX2 expression in ovarian cancer cell lines and decreased cell motility to various degrees.

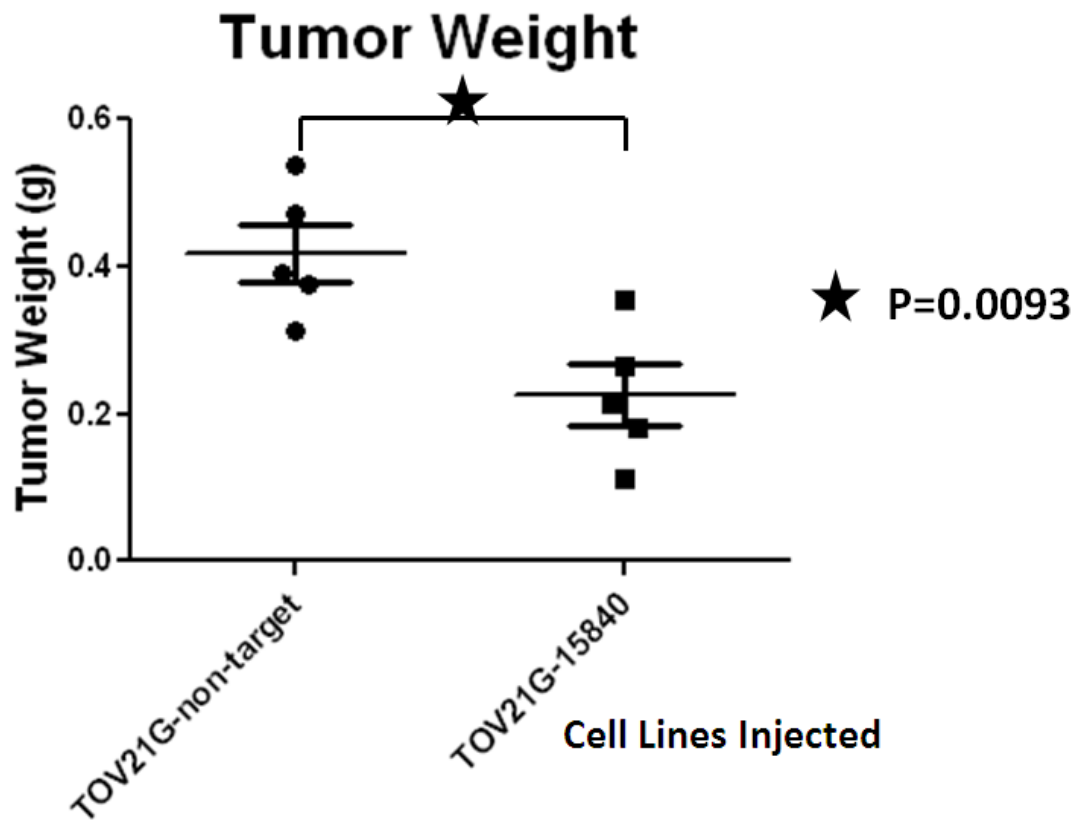


### **5.3.3 Knockdown PAX2 expression decreased tumor growth in *in vivo* experiments**

Ten nude mice were obtained from the Department of Experimental Radiation Oncology at The University of Texas MD Anderson Cancer Center. Ovarian cancer cell lines TOV21G-non-target control and TOV21G-PAX2KD-shRNA15840 were injected to these mice, five per group. Cells were dissolved in 2:1 PBS/matrix gel mixture. One million cells in 100µl PBS/matrix gel mixture were subcutaneously injected into each mouse left flank. Tumor size was measured at every four days before mice were sacrificed. We sacrificed the mouse after the average tumor volume was more than 0.5 cm<sup>3</sup> in any one group of mice. Tumors were weighed after the mice were sacrificed. Student's t-test was used to compare the tumor weight difference between two groups. We found that comparing to the control mice injected with TOV21G-non-target control cell line, mice injected with PAX2 silenced cell line TOV21G shRNA15840 had a lower tumor weight (P=0.0093) (Figure 5-3). This indicated that tumor grew slowly *in vivo* when we silenced PAX2 expression.

**Figure 5-3. Tumor grew slowly in mice injected with PAX2 silenced ovarian cancer cell lines.**

Tumor weight differences between mice injected with TOV21G-non-target control cell lines and TOV21G-PAX2KD-shRNA 15840 cell lines. Comparing to the control, mice injected with PAX2 silenced ovarian cancer cell lines had a lower tumor weight ( $P=0.0093$ ).



## **CHAPTER 6**

### **PAX2 KNOCKDOWN UPREGULATED G0S2 EXPRESSION AND ENHANCED APOPTOTIC EVENTS**



## 6.1 Introduction

*PAX2*, 5, 8 belong to the *PAX* gene family subgroup 2 (55) which are involved in a variety of tumor growth and survival (35). Bose (127) found that Angiotensin-II upregulated *PAX2* expression in prostate epithelial cells and prostate cancer cell lines results in increased cell growth. Stuart (99) found that *PAX2*, 5, 8 proteins can bind directly to the *TP53* gene 3' end of exon one and inhibit human *TP53* expression in astrocytoma. *PAX* proteins may regulate *TP53* expression at the transcriptional level and initiate tumor growth and progression. The relationship of *PAX2* and *PAX8* has mainly been studied in embryonic development. Batista (128) found *PAX8* spinal cord expression was regulated by *PAX2* in the embryonic spinal cord development and *PAX8* expression was dramatically downregulated in the absence of *PAX2*. Liu and Shan (13, 129) found that high-grade ovarian serous carcinoma commonly had *TP53* mutations, but such mutations were rare in low-grade ovarian carcinoma. Furthermore, low-grade serous carcinoma is characterized by mutations in the *KRAS* or *BRAF* pathway, as 68% of low-grade and 61% of LMP serous carcinomas have mutations in *KRAS* or its downstream mediator *BRAF* (13, 129). Thus, it is likely that the oncogenic *PAX2* promotes ovarian cancer development through a *TP53* dependent pathway, while *PAX8* which is expressed in *TP53* mutated high-grade ovarian cancer promotes the development of high grade ovarian cancer through a *TP53* independent pathway. Our study will be focused on *PAX2*'s function in different types of ovarian cancers, especially non-serous ovarian cancers which are more resistant to standard chemotherapy.

According to our literature research, we found that *PAX2* regulates the following genes: *MITF* (130), *ITGB3* (131), *ITGAV* (131), *WNT4* (132), *PAX2* (131, 133), *GCG* (134), *SFRP2* (135), *PAX5* (136), *VSX2* (130), *ROBO3* (137), *PAX6* (138), *GDNF* (139), *WT1* (133). *PAX2* is regulated by the following genes: *SHH* (140), *OTX2* (141-143), *Tlx* (144), *PAX2* (145), *ammonia* (146), *SIX1* (147, 148), *furosemide* (149), *HMX2* (150), *OTX1* (142), *GBX2* (142), *Akt inhibitor IV* (151), *BMP7* (152), *FST* (152), *VAX2* (153), and *HES3* (154). *PAX2* binds to the following genes: *WT1* (133), *GCG* (155, 156), *KPNA2* (157), *BBS1* (158), *BBS2* (158), *BBS4* (158), *BBS7* (158), *MAPK8* (159), *MAPK8IP1* (159), *PAXIP1* (160), *ID2* (161), *Rb* (137), *RBI* (137), *PAX5* (162), *PAX2* (162). Many of these genes are involved in apoptosis, cell motility and invasiveness, cell morphology change, cell growth and organ development, cell commitment and aggregation. Formerly, the research about *PAX2* is mainly focused on papillorrenal syndrome or renal-coloboma syndrome. The research about *PAX2* in ovarian cancers is still a blank until now. The role of *PAX2* in ovarian cancer is not well understood.

In humans, there are 12 different annexin proteins (annexins A1–A11 and A13) which have orthologues in most vertebrates (163). Annexin A1 (ANXA1) is a 37 kDalton protein formed by 346 amino acids which was previously known as lipocortin 1 (96). ANXA1 is the first member of 13 member protein family annexins which were first discovered by several laboratories when they were looking for the mechanisms of anti-inflammatory glucocorticoids suppressed prostanoid synthesis in cells (164-166). Annexin A1 signals through a seven-membrane spanning G-protein-coupled receptor (GPCR) (167) formyl peptide receptor 2 (FPR2) which is also known as ALXR in

humans. ALXR is also the receptor for the anti-inflammatory molecule lipoxin A4 (168). Human ALXR belongs to a small family of receptors which are consisted of three members (FPR1, ALXR and FPR3). These members are coupled to Gi proteins and are expressed by several cell types, such as human neutrophils, macrophages, monocytes, epithelial cells and endothelial cells (169, 170). Annexin A1 and peptides derived from its N-terminal region compete with ALXR ligand (serum amyloid protein A) and lipoxin A4 (167), for the binding site of ALXR. ANXA1 and lipoxin A4 (LXA4) analogs induce similar anti-inflammatory, protective effects by stimulating the lipoxin receptor LXA4 (ALX)/Formyl Peptide receptor-like 1 (FPRL-1) (171). ANXA1 is associated with many cellular components, including plasma membrane phospholipids, vesicles and cytoskeletal proteins and plays an important role in intracellular trafficking (94, 172). ANXA1 also plays an important role in cell differentiation, proliferation, apoptosis and plasma membrane repair intracellularly (94). ANXA1 is also a downstream mediator of glucocorticoid signaling, where it is an anti-inflammatory protein that has been implicated in the regulation of the mucosal regeneration during periods of inflammation in the host defense system (94, 173). Extracellularly, ANXA1 takes part in anti-inflammatory processes by inhibiting neutrophil adhesion to endothelial cell monolayers and decreasing transmigration, promoting neutrophils apoptosis and macrophage-regulated phagocytosis (168, 171, 174). We clarified the function of Annexin A1 in the development of ovarian cancer. Our data showed that PAX2 knockdown can decrease tumor growth by increasing

Annexin A1 expression and promoting apoptosis which is shown in our western blot analysis.

The Annexin V gene is located on human chromosome 4q26–q28 and spans a region of 28 kb DNA which contains 13 exons and 12 introns (175). Annexin V is a 35 kDa plasma protein which is expressed in syncytiotrophoblast and endothelial cells (176, 177). Recombinant exogenous Annexin V binds to negatively charged phospholipids in a  $\text{Ca}^{2+}$ -dependent manner, and this binding is important for its anticoagulant, antiapoptotic and anti-inflammatory effects (176-178). Munoz et al. hypothesized that Annexin V modulates the immune system by inhibiting phagocytosis of apoptotic and necrotic cells (179). Apoptotic cell death is characterized by a change in plasma membrane structure, the surface exposure of phosphatidylserine (PS), while the membrane integrity remains unchallenged. Surface exposed PS can be detected by its affinity to phospholipid binding protein Annexin V (180).

G0S2 protein was first identified as a small basic nuclear phosphoprotein which was encoded by G0S2, one of the G0/G1 switch (G0S) genes that are differentially expressed during lymphocytes lectin-induced switch from G0 to G1 phases of the cell cycle (86). The expression of G0S2 is required to commit cells to enter the G1 phase of the cell cycle (87). G0S2 is highly expressed in adipose tissue, liver and heart. Its expression increases in response to glucose, insulin and ligands for the PPAR family of transcription factors, and decreases upon treatment with  $\text{TNF}\alpha$  and  $\beta$ -adrenergic agonist (88, 89). G0S2 protein, a mitochondrial protein, specifically interacts with Bcl-2 and promotes apoptosis through preventing the formation of protective Bcl-2/Bax

heterodimers (90). DNA methylation of the G0S2 gene was significantly more frequent in squamous lung cancer than in non-squamous lung cancer (91) G0S2 with a functional PPRE (PPAR-responsive element) in its promoter is a direct PPAR $\gamma$  and probable PPAR $\alpha$  target gene and may be involved in adipocyte differentiation (92).

Stem cells were first described by Alexander Maksimov (181) as the common precursor cell of all blood cells. Stem cells are clonogenic, self-renewing progenitor cells which are able to generate one or more specialized cell types with the ability to differentiate into several different cells (182). Stem cells have the characteristics of self-renewal, proliferative capacity, and multipotency (183) and are classified into two major categories, according to their developmental status: embryonic stem cells and adult stem cells (182). Embryonic stem cells are pluripotent cells isolated from the inner cell mass of the blastocyst-stage mammalian embryo (184) and adult stem cells are found in adult tissues. Pluripotent cells can generate functional tissues during development and regenerate these tissues following injury or degenerative processes (182). Stem-cell fate is regulated by the combination of extrinsic and intrinsic signals, which are not fully understood (185). The molecular mechanisms that regulate stem-cell function and the identification of specific stem-cell markers will be the focus of cell and developmental biology (186, 187).

Somatic stem cells promote normal tissue repair and regeneration (188). Ovarian somatic stem cells within ovarian epithelial layer divide asymmetrically, yielding an undifferentiated self-cope and a daughter cell which proceeds to terminal differentiation (189). Multipotent ovarian somatic stem cells regulate ovarian surface epithelium repair

and regeneration after ovulatory rupture (188). Repeated asymmetric self-renewal increases the probabilities of mutations for somatic stem cells and their immediate progenitors, which ultimately lead to cancer stem cell formation and malignant progression (189).

Stem cells and tumor cells have several similarities and this led to the cancer stem cell model of tumorigenesis. Tumors are heterogeneous collections of rapidly proliferating cells with different phenotypes which is like organs derived from stem cells (183). These heterogeneous tumor cells are thought to derive from a self-renewing clonal population which produces terminally differentiating progenitor cells. This self-renewing clonal population within a tumor is termed “cancer stem cell” as they undergo similar biological processes as normal stem cells (183, 190). Cancer stem cells were first identified in acute myeloid leukemia by Lapidot etc. (191). They were subsequently identified in many types of solid tumors such as breast (192), ovarian (193, 194), prostate (195), brain (196), lung (197), liver (198), pancreas (199-201), colon cancer (202, 203), and melanoma (204). Cancer stem cells play a vital role in tumor initiation, growth, chemotherapy resistance and metastasis (186, 205, 206). Cancer stem cells are mainly responsible for early metastasis and recurrence (186, 205-207). They are also the key point for intrinsically resistance to chemotherapy (208) and radiotherapy (209) even after the disappearance of bulky tumors (210). BMI1, SHH, Notch and Wnt/beta-catenin pathways are characteristically activated in cancer stem cells (187, 211). Thorough research about these pathways will help us to identify potential therapeutic targets for cancer stem cells (187, 212).

Side population cancer cells is a part of cancer cells which is characterized by their ability to efflux lipophilic substrates, such as dye Hoechst 33342 and many chemotherapy agents(201). Side population cells which are immature, poorly differentiated, and highly tumorigenic have a higher expression of stem-cell markers and lower expression of differentiation markers in gene expression profiles (189).

## **6.2 Aims**

Downregulation of PAX2 can decrease cell growth. The mechanisms of this effect are our research focus. Affymetrix microarray analysis was used to study the genes that were affected by PAX2 knockdown. Ingenuity pathway analysis was used to study these differentially expressed genes. Taqman real-time RT-PCR was used to confirm our microarray data. RPPA was used to study proteins that were affected by PAX2 silencing. Cell cycle analysis, TUNEL, APC-Annexin V assay were used to study the apoptotic event changes affected by PAX2 silencing.

## **6.3 Results and Discussion**

### **6.3.1 PAX2 downregulation increased the expression of tumor suppressor gene *G0S2*.**

Expression profiles of genes affected by PAX2 knockdown were obtained by analysis of TOV21G cells with PAX2 knockdown by shRNA 15839, 15840, and 15841;

these profiles were compared with those of TOV21G, TOV21G-PLKO, TOV21G-non-target control cells. We found that silencing *PAX2* induced upregulation of *G0S2*, *WFDC1* and *GREM1* which are involved in the apoptotic events (Figure 6-1A, Table 6-1). Among the genes detected, *G0S2* was upregulated 3.85-fold compared with the non-target control in the TOV21G cell line (Figure 6-1A, Table 6-1). This change was confirmed by Taqman real-time RT-PCR (Figure 6-1B) in RMUGL, TOV21G, MDAH-2774, and IGROV1 ovarian cancer cell lines. In the mucinous ovarian cancer cell line RMUGL, transfection with shRNAs 15839, 15840, and 15841 caused *PAX2* downregulation ratios of 2.21, 1.64, and 3.56, respectively, and *G0S2* upregulation ratios of 2.30, 1.57, and 2.58 compared with the PLKO control. In the clear cell ovarian cancer cell line TOV21G, transfection with shRNAs 15839, 15840, and 15841 caused *PAX2* downregulation ratios of 4.62, 5.03, and 21.32, respectively, and *G0S2* upregulation ratios of 2.49, 5.31, and 3.57 compared with the non-target control. In the endometrioid ovarian cancer cell line MDAH-2774, transfection with shRNAs 15839, 15840, and 15841 caused *PAX2* downregulation ratios of 1.45, 1.56, and 3.60, respectively, and *G0S2* upregulation ratios of 1.84 and 1.58 compared with the non-target control. We did not see *G0S2* upregulation in the shRNA 15839 transfected MDAH-2774 cell lines. In the endometrioid ovarian cancer cell line IGROV1, transfection with shRNAs 15839, 15840, and 15841 caused *PAX2* downregulation ratios of *PAX2* of 1.45, 1.71, and 3.48, respectively, and *G0S2* upregulation ratios of 1.81, 2.85, and 3.77 compared with the non-target control (Figure 6-1B). In summary,



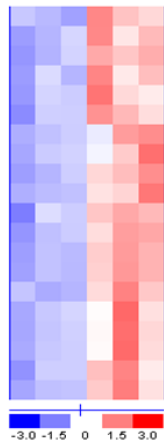
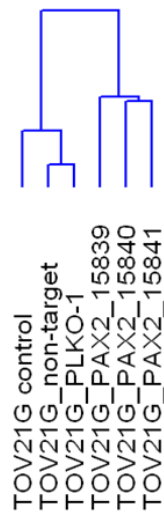
upregulation of *GOS2* was usually observed in the ovarian cancer cell lines that had downregulated *PAX2* expression.

We further analyzed our microarray data using Ingenuity Pathway Analysis (IPA) and found that the main biological functional pathways and canonical pathways had been changed obviously when we knockdown *PAX2* expression in the TOV21G ovarian cancer cell line. The most differentially expressed genes in the *PAX2* knockdown groups have their biological functions in the cellular movement, growth, proliferation, development, and tumor morphology and cell death (Figure 6-2A). These differentially expressed genes are mainly involved in the cancer development pathways, such as HER-2 signaling, estrogen receptor signaling, Wnt/ $\beta$ -catenin signaling, and cell cycle G1/S checkpoint regulation (Figure 6-2B). We further summarized these pathways affected by these differentially expressed genes in Figure 6-2C. These results further explained our observation that cell had decreased viability and mobility when we silenced *PAX2* expression and confirmed our hypothesis that *PAX2* is an oncogene.

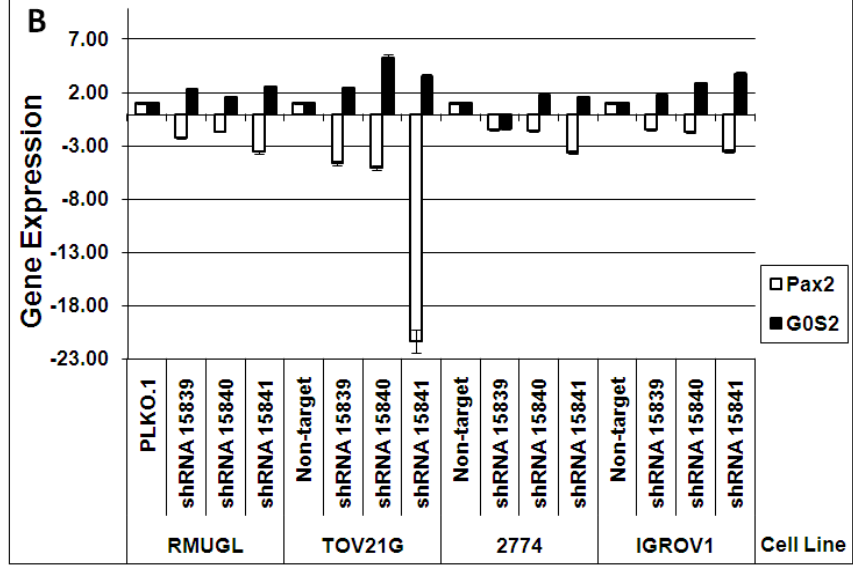
**Figure 6-1. *G0S2* was upregulated in *PAX2* stable knockdown ovarian cancer cell lines TOV21G, RMUGL, MDAH-2774, and IGROV1.**

(A) Differentially expressed genes in the *PAX2* stable knockdown TOV21G clear cell ovarian cancer cell line. (B) Validation of upregulation of *G0S2* in *PAX2* knockdown cell lines by RT-PCR. After *PAX2* knockdown in the TOV21G, Affymetrix microarray data showed that a list of genes (e.g., *G0S2*, *WFDC1*, and *GREM1*) had been upregulated. Taqman real-time RT-PCR further confirmed the overexpression of *G0S2* in *PAX2* knockdown ovarian cancer cell lines, TOV21G, RMUGL, MDAH-2774, and IGROV1.

**A**



C5orf13: chromosome 5 open reading frame 13  
AREG: amphiregulin  
MAP1B: microtubule-associated protein 1B  
PRSS23: protease, serine, 23  
LYPD1: LY6/PLAUR domain containing 1  
NKAIN4: Na<sup>+</sup>/K<sup>+</sup> transporting ATPase interacting 4  
GOS2: G0/G1 switch 2  
CRISPLD2: cysteine-rich secretory protein LCCL domain containing 2  
STC2: stanniocalcin 2  
STC2: stanniocalcin 2  
ABCA1: ATP-binding cassette, sub-family A (ABC1), member 1  
DCBLD1: discoidin, CUB and LCCL domain containing 1  
GREM1: gremlin 1, cysteine knot superfamily 1  
GREM1: gremlin 1, cysteine knot superfamily 1  
NFE2L3: nuclear factor (erythroid-derived 2)-like 3  
COL1A1: collagen, type I, alpha 1  
COL1A1: collagen, type I, alpha 1  
COL1A1: collagen, type I, alpha 1  
WFDC1: WAP four-disulfide core domain 1  
HS3ST3B1: heparan sulfate (glucosamine) 3-O-sulfotransferase 3B1

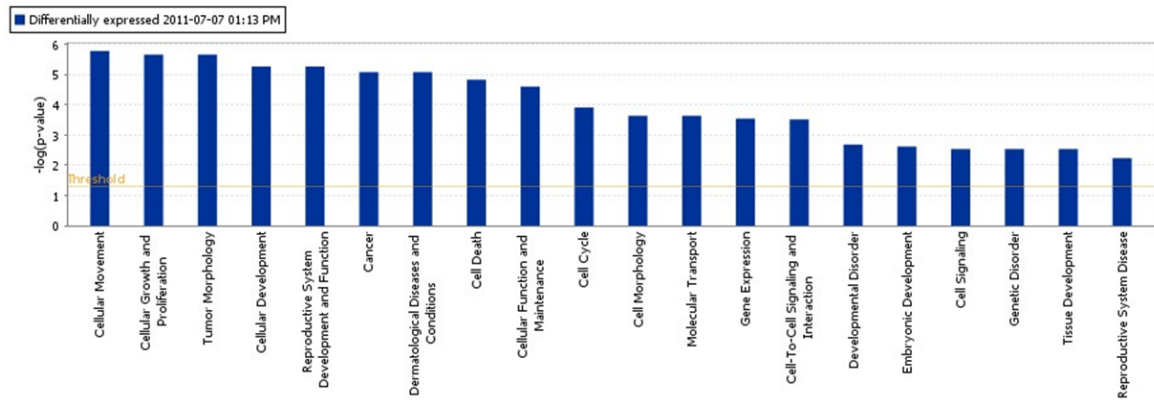


**Figure 6-2. Signaling pathways and biological functional changes induced by PAX2 downregulation.**

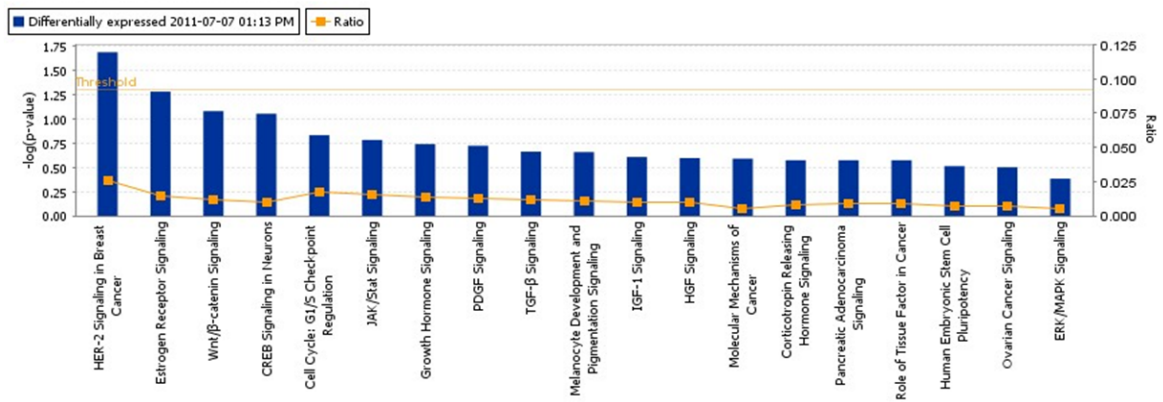
Ingenuity pathway analysis was used to analyze the biological functions and pathways affected by PAX2 knockdown. After downregulating PAX2 expression, differentially expressed genes were found by Affymetrix microarray analysis. Ingenuity pathway analysis was further used to analyze these differentially expressed genes.

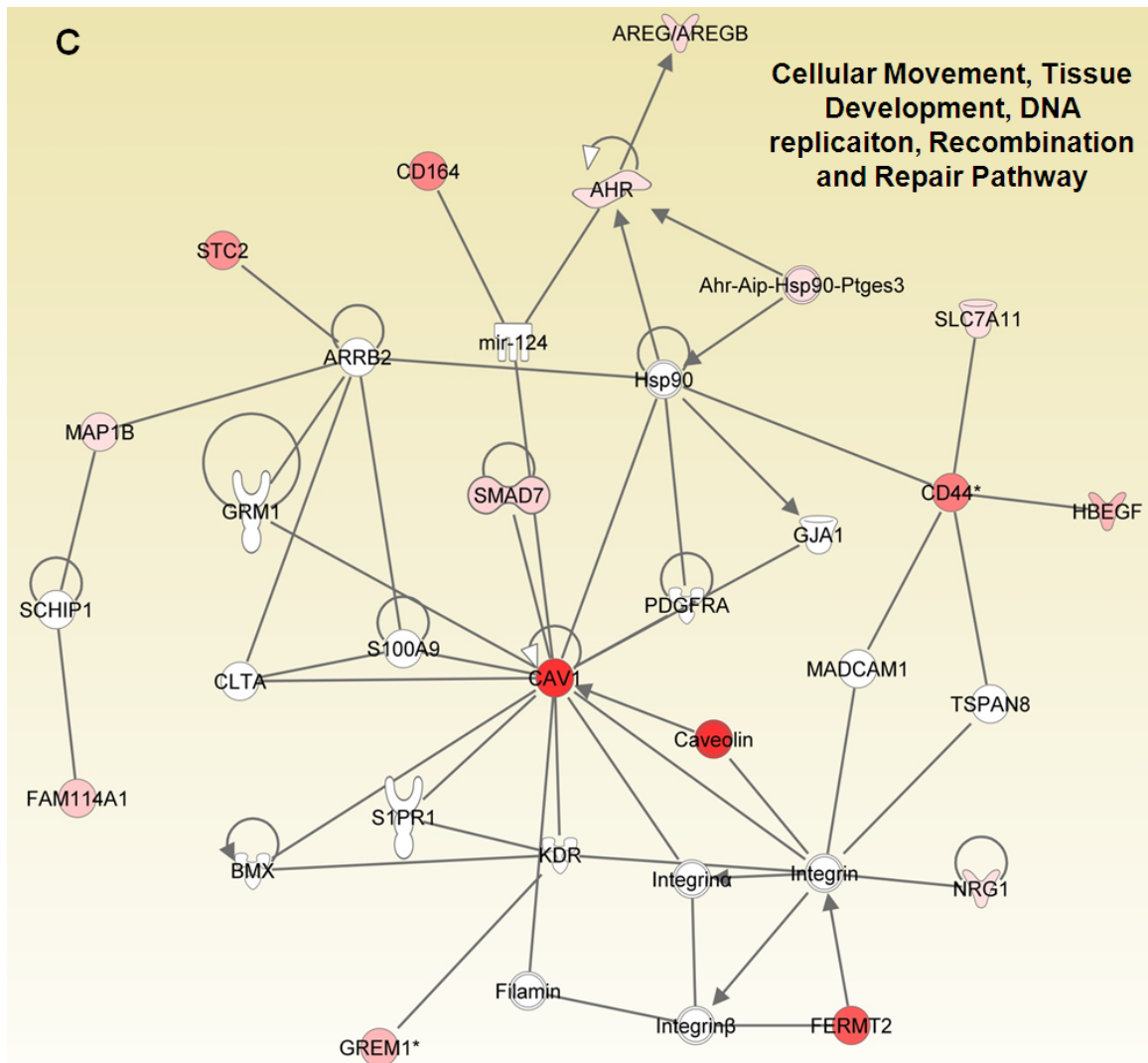
(A) Main biological functional changes affected by differentially expressed genes in the PAX2 stable knockdown TOV21G clear cell ovarian cancer cell line. (B) Main canonical pathways affected by differentially expressed genes in the PAX2 stable knockdown TOV21G clear cell ovarian cancer cell line. (C) Differentially expressed genes affect cellular movement, tissue development, DNA replication, recombination and repair pathway.

A



B





**Table 6-1. Differentially expressed genes between PAX2 knockdown cell lines and control cell lines in Affymetrix Microarray Analysis for clear cell ovarian cancer cell line TOV21G.**

Affymetrix microarray analysis was used to analyze gene expression differences between PAX2 positive control cell lines and PAX2 silenced cell lines in clear cell ovarian cancer cell line TOV21G. Genes that had been affected by PAX2 silencing were listed in this table.

Differentially expressed genes	Accession	EntrezGene	Description	baseline mean	experiment mean	fold change	lower bound of FC	upper bound of FC	difference of means	t statistic	P value	Biological Function
SLC7A11: solute carrier family 7, (cationic amino acid transporter, y+ system) member 11	AB040875	23657	g13516845	343.91	150.24	-2.29	-1.76	-2.88	-193.67	-4.194	0.044398	
PKDCC: protein kinase domain containing, cytoplasmic homolog (mouse)	BF528878	91461	Hs.10083.0	805.13	373.41	-2.16	-1.48	-3.37	-431.72	-3.324	0.044942	
TSPAN12: tetraspanin 12	AI056699	23554	Hs.16529.1	676.53	424.87	-1.59	-1.27	-2.01	-251.66	-3.367	0.043844	
DLST: dihydrolipoamide S-succinyltransferase (E2 component of 2-oxo-glutarate complex)	S72422	1743	Hs.296348.0	675.32	464.25	-1.45	-1.28	-1.65	-211.07	-4.786	0.017593	
GSTCD: glutathione S-transferase, C-terminal domain containing	AA740875	79807	Hs.44307.0	479.75	341.11	-1.41	-1.24	-1.58	-138.64	-4.107	0.039981	
CTBP1:C-terminal binding protein 1	BF984434		Hs.239737.1	811.05	589.91	-1.37	-1.27	-1.48	-221.14	-6.146	0.012158	Cellular Movement
Hs.47448.0	AW149422		Hs.47448.0	2661.68	1959.72	-1.36	-1.27	-1.45	-701.97	-7.195	0.007474	
Hs.172028.0	N51370		Hs.172028.0	2351.67	3005.77	1.28	1.2	1.36	654.1	6.669	0.006876	
SERINC5: serine incorporator 5	AI700633	256987	Hs.288232.0	746.98	979.37	1.31	1.2	1.44	232.39	5.572	0.014578	
ADCY7: adenylate cyclase 7	NM_001114	113	g4557254	377.65	497.1	1.32	1.23	1.42	119.46	6.976	0.00686	
PTPRK: protein tyrosine phosphatase, receptor type, K	NM_002844	5796	g4506316	630.78	870.44	1.38	1.23	1.57	239.66	4.94	0.022715	Cellular Movement
FERMT2: fermitin family homolog 2 (Drosophila)	Z24725	10979	g505032	2207.94	3080.68	1.4	1.23	1.61	872.75	4.566	0.021748	Cellular Movement
POLR2A: polymerase (RNA) II (DNA directed) polypeptide A, 220kDa	NM_000937	5430	g4505938	698.74	982.44	1.41	1.3	1.53	283.71	7.881	0.008744	
SLC20A1: solute carrier family 20 (phosphate transporter), member 1	NM_005415	6574	g7382462	1486.6	2156.85	1.45	1.29	1.65	670.25	5.883	0.016278	
CD164: CD164 molecule, sialomucin	BF669455	8763	Hs.43910.2	1536.92	2239.71	1.46	1.3	1.64	702.79	5.676	0.011666	
CD44: CD44 molecule (Indian blood group)	AF098641	960	g3832517	716.6	1044.17	1.46	1.21	1.79	327.57	3.55	0.038195	Cellular Movement
APBB2: amyloid beta (A4) precursor protein-binding, family B, member 2	BF115739	323	Hs.15740.0	332.91	488.78	1.47	1.23	1.79	155.87	3.924	0.033605	Cellular Movement
C5orf13: chromosome 5 open reading frame 13	U36189	9315	g1244509	255	373.82	1.47	1.21	1.85	118.81	3.442	0.04681	Cellular Movement
KLHL5: kelch-like 5 (Drosophila)	BE501881	51088	Hs.29088.0	457.38	675.14	1.48	1.25	1.76	217.76	3.862	0.04538	
NRG1: neuregulin 1	NM_013960	3084	g7669519	248.73	367.46	1.48	1.24	1.79	118.73	3.714	0.037901	Cellular Movement
SMAD7: SMAD family member 7	NM_005904	4092	g5174516	548.26	819.47	1.49	1.31	1.73	271.21	5.484	0.012297	Cellular Movement



SOCS6: suppressor of cytokine signaling 6	NM_016387	9306	g7705444	317.96	479.53	1.51	1.25	1.86	161.57	3.721	0.03556	
CD44: CD44 molecule (Indian blood group)	M24915	960	g180196	1560.1	2426.91	1.56	1.34	1.84	866.81	5.671	0.017411	Cellular Movement
CD44: CD44 molecule (Indian blood group)	BC004372	960	g13325117	1551.61	2425.07	1.56	1.32	1.9	873.46	4.842	0.019697	Cellular Movement
SOX9: SRY (sex determining region Y)-box 9	NM_000346	6662	g4557852	558.23	873.54	1.56	1.29	1.94	315.31	4.002	0.029238	
CAV1: caveolin 1, caveolae protein, 22kDa	NM_001753	857	g4580417	2413.45	3831.29	1.59	1.43	1.76	1417.84	6.746	0.041172	Cellular Movement
ETS2: v-ets erythroblastosis virus E26 oncogene homolog 2 (avian)	AL575509	2114	Hs.85146.0	188.74	304.16	1.61	1.34	2	115.42	4.482	0.020799	Cellular Movement
FAM114A1: family with sequence similarity 114, member A1	AI742174	92689	Hs.59622.0	623.38	1005.83	1.61	1.39	1.9	382.45	5.883	0.009804	
GALNT1: UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylglucosaminyltransferase 1 (GalNAc-T1)	NM_020474	2589	g13124890	428.38	708.79	1.65	1.49	1.85	280.41	7.81	0.011602	
AHR: aryl hydrocarbon receptor	NM_001621	196	g5016091	315.51	524.32	1.66	1.37	2.05	208.81	4.38	0.03393	
C20orf160: chromosome 20 open reading frame 160	BF970287	140706	Hs.118987.0	355.74	595.2	1.67	1.46	1.94	239.45	7.275	0.006084	
C5orf13: chromosome 5 open reading frame 13	NM_004772	9315	g4758865	591.21	1019.05	1.72	1.5	2.01	427.84	6.821	0.007469	Cellular Movement
GALNT1: UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylglucosaminyltransferase 1 (GalNAc-T1)	U41514	2589	g1136284	754.28	1298.95	1.72	1.43	2.15	544.67	5.714	0.017967	
CHST11: carbohydrate (chondroitin 4) sulfotransferase 11	AI806905	50515	Hs.64001.0	152.5	269.8	1.77	1.49	2.16	117.3	5.96	0.009469	
Hs.128842.0	AV712346		Hs.128842.0	154.51	273.05	1.77	1.36	2.5	118.54	4.121	0.040324	
CTSC: cathepsin C	AV699565	1075	Hs.128065.0	166.78	302.87	1.82	1.43	2.43	136.09	4.569	0.019761	
GALNT1: UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylglucosaminyltransferase 1 (GalNAc-T1)	BC038440	2589	Hs.2.80120.2	921.55	1689.21	1.83	1.61	2.12	767.66	9.217	0.003804	
SIX1: SIX homeobox 1	N79004	6495	Hs.41295.0	310.78	579.65	1.87	1.42	2.58	268.87	3.876	0.037635	Cellular Movement
FRMD6: FERM domain containing 6	AL040051	122786	Hs.250705.0	1142.29	2145.48	1.88	1.48	2.57	1003.19	5.279	0.029777	
GALNT1: UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylglucosaminyltransferase 1 (GalNAc-T1)	AV692127	2589	Hs.80120.0	687.75	1290.23	1.88	1.74	2.03	602.47	13.246	0.006358	

AREG: amphiregulin	NM_001657	374	g4502198	391.08	737.33	1.89	1.46	2.52	346.26	4.091	0.043639	Cellular Movement
FRMD6: FERM domain containing 6	N30138	122786	Hs.250705.0	993.83	1875.24	1.89	1.44	2.73	881.41	4.653	0.037928	
PRSS23: protease, serine, 23	AW471145	11098	Hs.25338.0	232.79	452.3	1.94	1.4	3.1	219.51	3.812	0.038181	
DCBLD1: discoidin, CUB and LCCL domain containing 1	N22751	285761	Hs.34665.0	244.83	502.36	2.05	1.72	2.54	257.53	8.387	0.005597	
PRSS23: protease, serine, 23	NM_007173	11098	g6005881	452.76	932.3	2.06	1.49	3.05	479.54	3.844	0.043023	
MAP1B: microtubule-associated protein 1B	AA554833	4131	Hs.113368.1	260.89	562.95	2.16	1.69	2.93	302.06	6.118	0.008805	Cellular Movement
HBEGF: heparin-binding EGF-like growth factor	NM_001945	1839	g4503412	620.03	1345.7	2.17	1.52	3.66	725.67	4.12	0.027068	Cellular Movement
CHST11: carbohydrate (chondroitin 4) sulfotransferase 11	AI123348	50515	Hs.64001.0	174.99	381.05	2.18	1.56	3.43	206.06	4.374	0.022198	
ST3GAL5: ST3 beta-galactoside alpha-2,3-sialyltransferase 5	NM_003896	8869	g4506954	337.17	753.37	2.23	1.69	3.18	416.21	5.308	0.014814	
STC2: stanniocalcin 2	AI435828	8614	Hs.155223.0	841.66	2018.28	2.4	1.89	3.1	1176.62	5.933	0.037157	
NKAIN4: Na+/K+ transporting ATPase interacting 4	AI758937	128414	Hs.46627.0	160.16	399.78	2.5	1.63	4.85	239.62	4.036	0.027952	
ABCA1: ATP-binding cassette, sub-family A (ABC1), member 1	AF285167	19	g9755158	108.08	321.09	2.97	1.78	8.69	213.01	4.674	0.029427	
LYPD1: LY6/PLAUR domain containing 1	AL567376	116372	Hs.85339.1	254.22	758.29	2.98	1.75	9.34	504.07	4.308	0.02723	
G0S2: G0/G1switch 2	NM_015714	50486	Hs.95910.0	268.07	1673.14	6.24	4.2	12.01	1405.07	14.098	0.000825	
GREM1: GREMlin 1, cysteine knot superfamily, homolog (Xenopus laevis)	AF154054	26585	g10863087	182.53	1338.66	7.33	4.21	26.12	1156.13	9.787	0.004685	Cellular Movement
GREM1: GREMlin 1, cysteine knot superfamily, homolog (Xenopus laevis)	NM_013372	26585	g7019348	112.29	930.82	8.29	4.68	31.71	818.53	9.507	0.010272	Cellular Movement

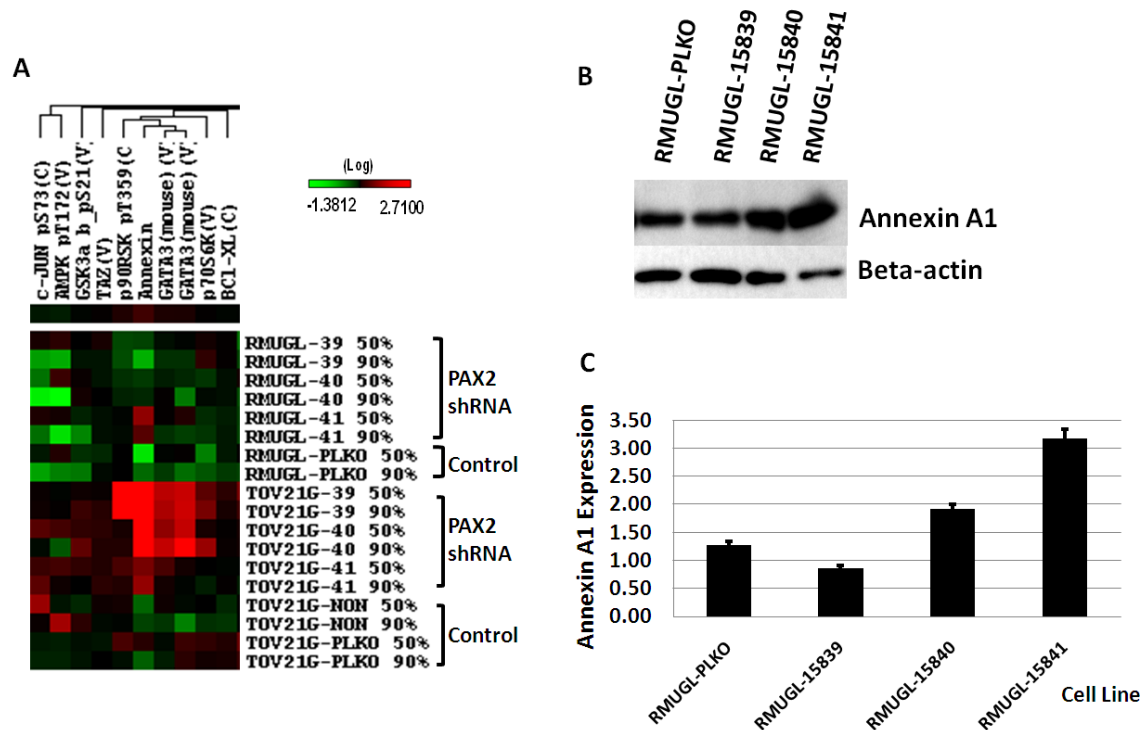
### **6.3.2 PAX2 downregulation in ovarian cancer cell lines promoted apoptotic events.**

RPPA was used to analyze the effects of PAX2 knockdown in ovarian cancer cell lines RMUGL and TOV21G. RPPA sample preparation, slide printing, staining and data analysis were processed as described (213, 214). Two hundred seventeen antibodies were used to probe our samples. Of these, 207 antibodies functioned adequately in the array and were used in the analysis. Knockdown of PAX2 expression increased the expression of Annexin A1, a marker of early stage apoptosis (Figure 6-3A). Annexin A1 expression was further confirmed using western blot analysis (Figure 6-3B, Figure 6-3C). To confirm PAX2's function in the early stage of apoptosis, allophycocyanin-Annexin V staining, which detects an increase of phosphatidylserine residues in the outer plasma membrane leaflet during the early stages of apoptosis was used to measure apoptotic cells in RMUGL and TOV21G ovarian cancer cell lines with or without PAX2 expression (Figure 6-4, Table 6-2). In RMUGL, the percentage of apoptotic cells was 6.73%, 9.01%, and 17.15% for cells transfected with shRNAs 15839, 15841, and 15841, respectively, compared with 8.95% in the PLKO control (Figure 6-4A). In TOV21G, the percentage of apoptotic cells was 3.48%, 5.27% and 7.63% for cells transfected with shRNAs 15839, 15841, and 15841, respectively, compared with 3.47% in the PLKO control (Figure 6-4B, Figure 6-4C). Compared with the PAX2-expressing ovarian cancer cell line controls, PAX2 knockdown ovarian cancer cell lines had an increase in the percentage of Annexin V-positive apoptotic cells (Figure 6-4). The RPPA and Annexin V assay data were consistent with each other. We also found that in our RPPA data, silencing PAX2 expression did not affect the later

stage of apoptosis which is marked by increased TP53 expression. We verified this using western blot analysis to check TP53 expression (data not shown). We found that silencing PAX2 expression had no effect on the TP53 expression.

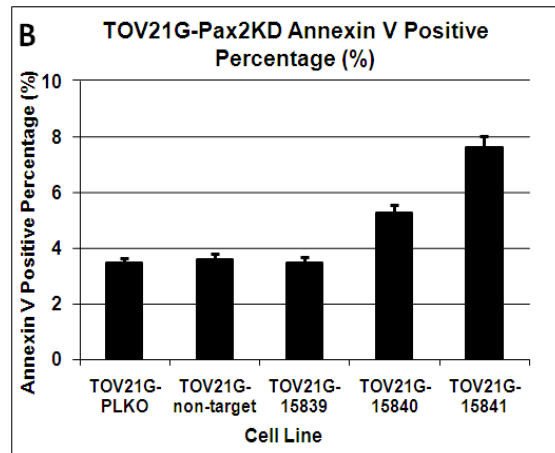
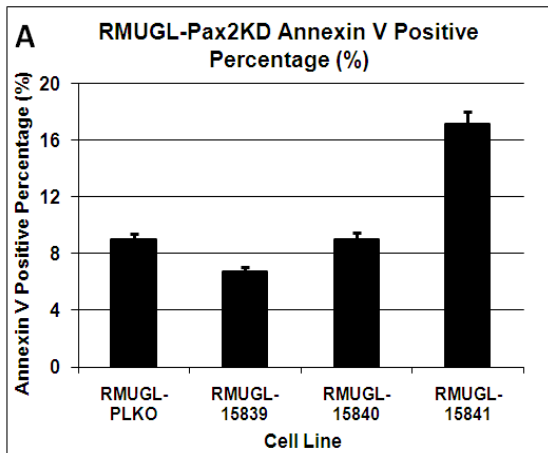
**Figure 6-3. PAX2 knockdowns in ovarian cancer cell lines TOV21G and RMUGL affect proteins involved in apoptosis.**

Upregulation of Annexin A1 in PAX2 stable knockdown ovarian cancer cell lines. (A) Reverse phase protein array (RPPA) analysis showed PAX2 knockdown ovarian cancer cell lines had a higher expression of Annexin A1 than control cells. (B) Western blot analysis was used to measure Annexin A1 expression in RMUGL ovarian cancer cell lines with or without PAX2 knockdown. Compared with the control PLKO, PAX2 knockdown ovarian cancer cell lines had higher expression of Annexin A1, which further confirmed our RPPA results. (C) Western blot results normalization for Annexin A1 expression in mucinous ovarian cancer cell line RMUGL.

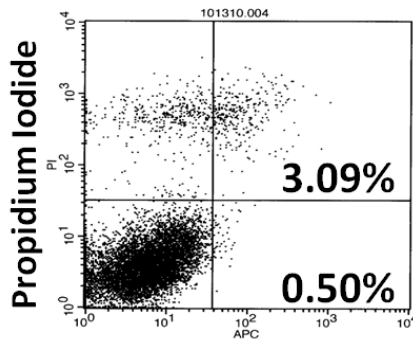


**Figure 6-4. Allophycocyanin-Annexin V staining in PAX2 knockdown ovarian cancer cell lines.**

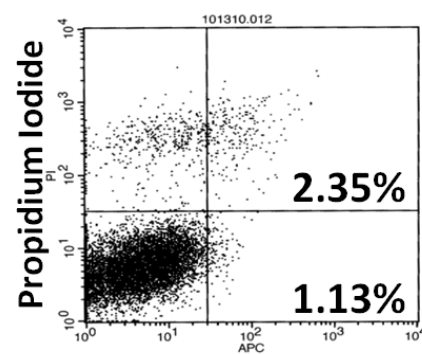
Flow cytometric analysis of cell apoptosis using APC-Annexin V staining. (A) Mucinous ovarian cancer cell line RMUGL. (B) Clear cell ovarian cancer cell line TOV21G. (C) Examples of Annexin V staining of clear cell ovarian cancer cell line TOV21G. Compared with control cells, PAX2 knockdown ovarian cancer cells had a higher percentage of apoptotic cells.



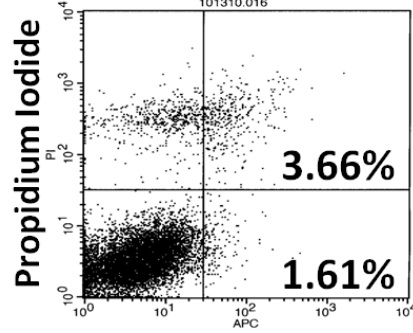
**C** TOV21G non-target shRNA



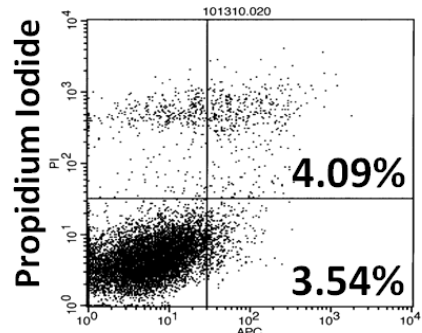
TOV21G PAX2 shRNA 15839



TOV21G PAX2 shRNA 15840



TOV21G PAX2 shRNA 15841



Annexin V

Annexin V

**Table 6-2. Downregulating PAX2 expression in ovarian cancer cell lines promoted apoptotic events as revealed by Allophycocyanin-Annexin V staining.**

Summary of Annexin V positive percentage in cell lines with/without PAX2 knockdown.

Cell Line Name	Annexin V+(%)	Annexin V& PI+(%)	Total Annexin V+(%)
RMUGL-PLKO	1.30	7.65	8.95
RMUGL-15839	2.05	4.68	6.73
RMUGL-15840	0.98	8.03	9.01
RMUGL-15841	7.73	9.42	17.15
TOV21G-PLKO	0.57	2.90	3.47
TOV21G-non-target	0.50	3.09	3.59
TOV21G-15839	1.13	2.35	3.48
TOV21G-15840	1.61	3.66	5.27
TOV21G-15841	3.54	4.09	7.63



### **6.3.3 PAX2 did not promote ovarian cancer growth by affecting later stage of apoptosis.**

After successfully silencing PAX2 expression in ovarian cancer cell lines, TOV21G, RMUGL, MDAH-2774 and IGROV1, we compared the cell cycle changes and apoptotic differences between ovarian cancer cell lines with PAX2 expression and PAX2 silenced ovarian cancer cell lines using cell cycle analysis and TUNEL assay. We found that these two kinds of cell lines had similar cell cycle pattern and the apoptotic cell numbers are similar. The difference had no statistic significance. This is consistent with our RPPA data which showed that silencing PAX2 expression had no effect on TP53 expression. This indicated that PAX2 might affect cell growth and motility through some other pathways instead of regulating cell cycle.

### **6.3.4 PAX2 did not promote ovarian cancer growth by affecting stem cells.**

Side population analysis was used to compare putative stem cell ratio between PAX2 expressed ovarian cancer cell lines and PAX2 silenced ovarian cancer cell lines. Cells were labeled with Hoechst 33342 dye (Invitrogen) and analyzed by using BD LSRII System (BD Biosciences). We did not find side population in RMUGL ovarian cancer cell lines. We did find side population in TOV21G ovarian cancer cell lines, but we did not find any difference between PAX2 expressed control TOV21G ovarian cancer cell lines and PAX2 silenced TOV21G ovarian cancer cell lines (data not shown). Thus, PAX2 did not promote ovarian cancer growth by affecting stem cells number.

## **CHAPTER 7**

### **DISCUSSION, FUTURE DIRECTIONS AND SIGNIFICANCE**

## 7.1 Discussion

*PAX* (paired box) genes were first described by Markus in 1986 (28) as a conserved sequence in the *Drosophila paired* and *gooseberry* genes. The nine members of human *PAX* gene family share a 384 base pair highly conserved DNA sequence, the paired box (32). Each human *PAX* gene is located on a different chromosome which suggests that they act singularly and not in combination (33). *PAX* genes regulate tissue development and cellular differentiation in embryos by promoting cell proliferation, cell-lineage specification, migration and survival (35, 36). *PAX* genes also regulate organogenesis and its mutations cause significant developmental abnormalities in a broad spectrum of organisms from flies to humans (49, 50). Their inactivation generally results in embryonal or neonatal death accompanied by striking developmental defects (38). In most cases, *PAX* gene expression attenuates when development is complete (34). Unattenuated *PAX* gene expression in adult tissues directs organ-specific regenerative events (51) and protects against stress-induced cell death (52). *PAX* genes are involved in stem-cell self-renewal, both during fetal development and in adult life (35). Cancer cells may signal through the *PAX* gene pathway to undergo proliferation, stem-cell self-renewal, resistance to apoptosis, cell migration and invasion which contributes to the malignant growth suggested by Hanahan and Weinberg (35, 53, 54). This indicates that *PAX* genes are proto-oncogenes (55) and deregulation of *PAX* genes contributes to tumor formation (56).

Our former research (111) found that *PAX2* is one of the most upregulated genes in low-grade ovarian serous carcinoma by gene expression profiling. We also

validated this result by real-time RT-PCR, western blot and immunohistochemical analysis which was the first time for PAX2 to be studied in ovarian cancer (111). Subsequently, we evaluated gene expression profiles on twenty-one low-grade serous ovarian cancer samples, twenty-four high-grade serous ovarian cancer samples and six normal ovarian surface epithelia (HOSE) samples from The University of Texas MD Anderson Cancer Center using Affymetrix microarray analysis. We found eight highly overexpressed genes and fourteen significantly underexpressed genes in low-grade serous ovarian cancer compared to high-grade serous ovarian cancer. PAX2 was one of the overexpressed genes with 4.78 times higher expression compared to high-grade samples with a p-value of 0.04. To verify PAX2 expression in different types of ovarian cancer samples, we did the immunohistochemical analysis using twenty-four clear cell ovarian cancer tissue paraffin sections and fifty-one mucinous ovarian cancer tissue paraffin sections. Tumor sections were either lack of nuclear staining or had robust nuclear staining. No weak or moderate strength staining was observed. The number of PAX2 positive tumor cells varied from 1-100%. In twenty-four clear cell ovarian cancer tissue sections, 20.8%, 41.7% and 20.8% were 1-25%, 25-50%, and 75-100% positive respectively. In fifty-one mucinous ovarian cancer tissue sections, 24.3%, 28.6% and 20% were 1-25%, 25-50%, and 75-100% positive respectively. In our former research, about 263 high-grade serous ovarian carcinoma tissue paraffin sections, 2%, 5% and 3% were  $\leq 1\%$ , 10-50%, and 75-100% positive respectively. In conclusion, non-serous ovarian cancer tissue sections showed robust nuclear staining of PAX2. This is consistent with and further confirmed our former data about PAX2 expression in

ovarian cancers (111). Considering that non-serous ovarian cancers are relatively resistant to standard chemotherapy, it is imperative for us to find a better method to treat these kinds of tumors. PAX2 may be a potential therapeutic target for non-serous ovarian cancers.

We screened twenty-eight human ovarian carcinoma cell lines (eight serous ovarian cancer cell lines, twelve clear cell ovarian cancer cell lines, three mucinous ovarian cancer cell lines, five endometrioid ovarian cancer cell lines) and one immortalized ovarian surface epithelium cell line IOSE29 for PAX2 nuclear expression by using western blot analysis. We also used Taqman real-time RT-PCR to confirm our western blot results at the mRNA level. We found that fourteen ovarian cancer cell lines, OVCAR3, OVCA432, TOV21G, TOV112D, RMUGL, HCH, KF, KOC7C, OVAS, OVISE, OVSAYO, MDAH-2774, IGROV1 and OVTOKO, had detectable higher PAX2 expression from 2 to 3220 folds comparing to the reference RNA. This was consistent with the western blot results. These PAX2 positive cell lines are mainly non-serous ovarian cancer cell lines. The cell line screening results were consistent with the human samples' results as to the PAX2 expression. We used these cell lines to do the PAX2 functional study.

Ovarian cancer cell lines TOV21G, RMUGL, MDAH-2774 and IGROV1 had obvious PAX2 expression in both western blot analysis and Taqman real-time RT-PCR and were used for PAX2 silencing experiments and functional studies. We used MISSION TRC shRNA Lentiviral Particles to transfect the ovarian cancer cell lines, TOV21G, RMUGL, MDAH-2774 and IGROV1. We first did the sequence blast for the

shRNA lentiviral particles to assure that this shRNA will only target PAX2. After stable knockdown PAX2 expression, we used western blot analysis to verify the efficiency of knocking down PAX2 at the protein level and used Taqman real-time RT-PCR to confirm PAX2 knockdown at the mRNA level. We also checked other PAX family members, such as PAX8 and found that PAX8 expression was not affected by PAX2 knockdown. Our PAX2 mission shRNA lentiviral particle transduction was efficient and specific.

After successfully silencing PAX2 expression in ovarian cancer cell lines, RMUGL, TOV21G, MDAH-2774 and IGROV1, we did WST1 assay on both the original ovarian cancer cell lines and PAX2 silenced cell lines. As shown in Figure 5-1A, comparing to the PLKO control cell line, all the PAX2 silenced RMUGL cell lines had a decreased proliferation rate. The same was observed in clear cell ovarian cancer cell line TOV21G (Figure 5-1B), endometrioid ovarian cancer cell lines MDAH-2774 (Figure 5-1C) and IGROV1 (Figure 5-1D). This indicated that PAX2 silenced cell lines had a lower proliferation rate compared with the PAX2 positive control cell lines. The consequence of lower proliferation rate is a slower cell growth rate. Thus, knockdown PAX2 expression decreased cell growth in ovarian cancer cell lines and this is consistent with Khoubehi's results that *PAX2* may be a proto-oncogene (79). This is also consistent with Bose's research for prostate cancer which showed that Angiotensin-II upregulated PAX2 expression in prostate epithelial cells and prostate cancer cell lines resulted in increased cell growth (127). We concluded that PAX2 downregulation decreased ovarian cancer cell growth by decreasing cell viability.

We also performed the wound healing assay to analyze the effects of silencing PAX2 expression in ovarian cancer cell lines. We used ovarian cancer cell lines, RMUGL, TOV21G, MDAH-2774 and IGROV1. After stable knockdown PAX2 expression in these cell lines, the cultures were photographed at 0h, 5h, 10h and 24h respectively. We used TScratch program to measure the open areas. We found that compared with control cell lines which had PAX2 expression, PAX2 silenced cell lines had bigger open areas and lower cell motility as shown in figure 5-2 which indicated that silencing PAX2 expression decreased cell motility.

Mouse model was also used to confirm PAX2's function in *in vivo* experiments. Ten nude mice were injected with either ovarian cancer cell lines TOV21G-non-target control or TOV21G-PAX2KD-shRNA15840, five per group. Tumor size was measured at every four days until the average tumor volume was more than 0.5 cm<sup>3</sup> in any one group. Tumor weight was measured after the mice were sacrificed. We found that compared with the control mice injected with TOV21G-non-target control cell line, mice injected with PAX2 silenced cell line TOV21G shRNA15840 had a lower tumor weight (P=0.0093) (Figure 5-3) which indicated that tumor grew slowly *in vivo* when we silenced PAX2 expression.

To verify the pathways involved in the development of ovarian cancer, we used Affymetrix microarray analysis to compare the gene expression differences between PAX2 positive control cell lines and PAX2 silenced cell lines in clear cell ovarian cancer cell line TOV21G. We found that silencing PAX2 expression induced upregulation of tumor suppressor gene *G0S2* (Figure 6-1A). We verified our microarray

data using Taqman real-time RT-PCR in four ovarian cancer cell lines, RMUGL, TOV21G, MDAH-2774 and IGROV1. We found that Taqman real-time RT-PCR results were consistent with the microarray results (Figure 6-1B). PAX2 downregulation increased the expression of tumor suppressor gene *GOS2*. We did not find changes in the other PAX gene family members and this had excluded the most possible off-target effects.

We also checked a list of genes that were involved in the different pathways, such as *AKT*, *PI3K*, EMT and cell dedifferentiation. Our data showed that silencing PAX2 expression had no effect on these pathways. We further verified the effects of silencing PAX2 expression on different pathways using reverse phase protein array analysis. We probed samples with 217 antibodies. Based on our QC samples, we deleted those antibodies that do not function well in the array. We included 207 antibodies in the data set. We found that among these proteins, Annexin A1 had a prominent increase when we silenced PAX2 expression. We did not find changes in the other PAX gene family members which further confirmed that our shRNA lentiviral transduction system was specific. We further used western blot analysis to confirm our RPPA data. Since Annexin A1 is a marker for apoptosis, our data indicated that PAX2 downregulation upregulated Annexin A1 expression and promoted apoptosis. Thus, PAX2 might affect cell growth by affecting apoptosis.

We further confirmed PAX2's function in the early stage of apoptosis by APC Annexin V staining. Apoptotic cell death is characterized by complete membrane integrity with the surface exposure of phosphatidylserine which can be detected by its



affinity to phospholipid binding protein Annexin V using APC-Annexin V staining (180). We found that comparing to the PLKO and non-target control, the PAX2 silenced cell lines had an increased percentage of Annexin V positive cells in both RMUGL and TOV21G cell lines (Figure 6-2E, Figure 6-2F) which indicated that knockdown PAX2 expression induced the early stage of apoptosis.

TP53 is a marker for the later stage of apoptosis and TUNEL assay can be used to examine the later stage of apoptosis. We checked TP53 expression in both PAX2 positive cell lines and PAX2 silenced cell lines and found that silencing PAX2 expression had no prominent effect on TP53 expression. This was consistent with our cell cycle analysis and TUNEL assay results, which indicated that PAX2 did not affect cell growth by affecting the later stage of apoptosis.

In summary, cancer cells may signal through the *PAX2* pathway to proliferate, develop resistance to apoptosis, and promote cell migration and invasion, which contributes to the malignant growth as suggested by Hanahan and Weinberg (35, 53, 54). *PAX2* therefore may be a potential therapeutic target for non-serous ovarian cancers which are resistant to standard chemotherapy.

## **7.2 Future directions and significance**

*PAX2* belongs to the *PAX* gene subgroup II which is involved in embryonic development (68) and tumor growth (35, 69). The human *PAX2* gene is located on chromosome bands 10q24.3-10q25.1 (70) and composed of 12 exons which spans approximately 70 kb (71). This gene encodes a 48-50 KDa PAX2 (64) transcription

factor (46), which regulates the differentiation of urogenital system, eyes, ears, and central nervous system during the early development (69, 72). The *PAX2* gene is frequently expressed in a panel of 406 common primary tumor tissues (97) and its expression is significantly higher in patients with metastatic disease as it is correlated with the proliferation index (98). *PAX2* promotes the cancer cell survival, motility and growth (35) and serves as a critical component of the multi-step oncogenic transformation process and is a likely candidate (65) for oncogenesis by stimulating proliferation (72, 99, 100). *PAX2* may be a potential cancer therapeutic target as it suppresses cisplatin-induced apoptosis and the silencing of *PAX2* partially overcomes the resistance of renal cell carcinomas to chemotherapy *in vivo* (101). Yet, little is known about the mechanism of *PAX2*-mediated protection from cell death (66). Currently, the research about *PAX2* is mainly focused on embryonic development and kidney carcinoma. Our study about *PAX2*'s function in the ovarian cancer is a new milestone for both *PAX2* research and ovarian cancer research. As we know, ovarian cancer is the second most common gynecologic malignancy, and the most common cause of death among women who develop gynecologic cancers (5). It is also the fifth most common cause of cancer-related death in females in the United States (5). It is called a "silent killer" (7) because in the early stage, the symptoms are vague, non-specific (8) and the obvious symptoms can only be found when the disease has advanced and the treatment is unfavorable. About 85% of ovarian cancers are low grade ovarian cancers which are normally undetectable clinically. Conventional treatment for ovarian cancer is surgery, especially for early stage ovarian tumors for which only the

involved ovary and fallopian tube are removed (known as "unilateral salpingo-oophorectomy," USO) (15); this is suitable for young women who wish to preserve their fertility. For later stage ovarian cancer, the combination of surgery and chemotherapy is necessary. First-line chemotherapy with platinum drugs (e.g. cisplatin and carboplatin) and taxanes (e.g. paclitaxel) yields a response rate of more than 80% (16); however, nearly all patients relapsed and the side effect is obvious and serious. Thus, many targeted therapies use non-chemotherapy drugs to target specific cancer cells are in development. Unlike chemotherapy, targeted therapy spares normal cells, and may reduce the side effects of other therapies. Our research data provide the theory basis for ovarian cancer target therapy. Although targeting a transcription factor using small molecule is not very feasible, recent identification of an immunogenic HLA-A\*0201-binding T-cell epitope of the transcription factor PAX2 provides a promising view for cancer immunotherapy (24). We expect to see the PAX2 targeting for ovarian cancer immunotherapy in the near future.

In summary, we demonstrated that PAX2 knockdown in ovarian cancer cells suppressed cell proliferation and motility. Stable PAX2 knockdown upregulated the tumor suppressor gene *G0S2*, and promoted the early stages of apoptosis. We found that clear cell, endometrioid, and mucinous cell ovarian cancers had a higher expression of PAX2 compared with high-grade serous ovarian cancers; therefore, targeting the PAX2 pathway in these ovarian cancers warrants further investigation. Our research is focused on the molecular mechanisms of *PAX* genes' function and its downstream target genes, which contributed to both the developmental processes and the molecular

mechanisms underlying pathogenesis of cancer (55). Furthermore, our research also provides the theoretical basis for ovarian cancer immunotherapy by targeting PAX2 gene (24).

Further research will be focused on finding the exact binding site of PAX2 on *G0S2* promoter and its exact binding sequence. This will provide a promising aspect for ovarian cancer immunotherapy by targeting PAX2. We can first knockdown *G0S2* in the parental cell lines with PAX2 expression and then knockdown PAX2 expression in these cell lines without *G0S2* expression. Then, we can check the cell viability and motility. If our hypothesis that PAX2 regulates cell growth through downregulating *G0S2* expression is correct, we should not see the growth inhibition effects of PAX2 Knockdown among these cell lines. We can further check about the binding sites between PAX2 and *G0S2*. In fact, our ExPlain software analysis had given a 10.5145 sites/1000bp binding match with a P-value of 0.0036 which showed a very high binding possibility between PAX2 protein and *G0S2* promoter regions. Our hypothesis that PAX2 binds to the promoter region of *G0S2* and downregulates *G0S2* expression can be confirmed by chromatin immunoprecipitation (ChIP) assay.

We further did the ExPlain analysis for *ANXA1* gene which encodes Annexin A1 protein. We did not find any binding possibility between them which means that it is rarely possible for PAX2 to bind to the *ANXA1* promoter or for *ANXA1* to bind to the Pax2 promoter. There is no meaning of doing ChIP analysis for them. If we want to check protein level binding possibility, we need to do mass spectrometry analysis and find the potential binding sites. But, in my opinion, there may be some other pathways

between these two genes and they may not bind directly according to the preliminary literature search.

Another direction of our study will be focused on further investigating IPA signaling pathway data. We can check their binding possibilities with PAX2 and knockdown the high possible binding genes and figure out new pathways involved in the cancer development and progression. This will be a profound contribution to the present cancer research and may open a new chapter for them.

The most promising part of our future research will be the drug resistance study which is directly linked to the clinical patient treatment. We can treat the cell lines with/without PAX2 expression using present chemotherapy drugs, such as platinum drugs (e.g. cisplatin and carboplatin) and taxanes (e.g. paclitaxel); and observe cancer cells response to the chemotherapy. If the cell viability and motility difference between the same ovarian cancer cell lines with/without PAX2 expression changed greatly after chemotherapy, we may conclude that PAX2 affects cells' sensitivity to the chemotherapy. We can further use mouse xenograft model to prove that PAX2 may be related to drug resistance. Chemotherapy drugs can be used to treat mouse injected with ovarian cancer cell lines with/without PAX2. If the tumor size and weight differences between mice injected with ovarian cancer cell lines with/without PAX2 expression changed greatly, our hypothesis that PAX2 is related to drug resistance will be further confirmed.

The most obvious limitation of our study was that we used xenograft mouse model to study PAX2's effect on the development and progression of ovarian cancer.

For xenograft ovarian cancer mouse model, we injected human ovarian cancer cell lines subcutaneously into the mouse's flank. This model needs less work and the tumor forming time is short. It is a suitable way to evaluate chemotherapy drugs before the large scale clinical usage. But, xenograft mouse model doesn't consider the tumor and microenvironment interactions which is very vital for the tumor development and progression. Many tumors can have metastasis if we use orthotopic mouse model instead of xenograft model. Thus, we need to prove our hypothesis using orthotopic model, especially considering that PAX2 may affect metastasis according to our wound healing assay.

Orthotopic ovarian cancer mouse model which implants tumor to the ovary can modulate the real ovarian cancer development much better by simulating tumor morphology, microenvironment, metastasis potential and drug responses. Thus, we need to use orthotopic ovarian cancer mouse model to further verify our present results of xenograft ovarian cancer mouse model. This will be another focus of our future research.

In summary, we observed PAX2's expression and function and validated a mechanism for PAX2's function in the ovarian cancer development and progression. These studies provided the theoretical basis for clinical cancer treatment and had profound impact on the future ovarian cancer research.

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