Emerging Roles for PAX8 in Ovarian Cancer and Endosalpingeal Development

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ABSTRACT

Objectives.
Epithelial ovarian carcinomas develop from ovarian surface epithelia that undergo complex differentiation to form distinguishable phenotypes resembling those of the epithelia of the female urogenital regions. While previous studies have implicated regulatory developmental homeobox (HOX) genes in this process, other factors responsible for this differentiation are largely unknown. Aberrant transcriptional expression of PAX8 has been reported in epithelial ovarian cancer, prompting us to initiate the molecular characterization of this master regulatory gene in ovarian cancer development.

Methods.
Immunohistochemistry, immunoblotting and RT-PCR were used to investigate the presence of PAX8 and its protein products in epithelial ovarian cancer subtypes, normal ovarian surface epithelia, ovarian inclusion cysts, and normal endosalpingeal epithelia.

Results.
In this report, we confirm microarray results indicating that the transcription factor, PAX8, is highly expressed in epithelial ovarian cancer but absent from the precursor ovarian surface epithelia of healthy individuals. Furthermore, we report that PAX8 is localized to the nucleus of non-ciliated epithelia in simple ovarian epithelial inclusion cysts and in three epithelial ovarian cancer subtypes (serous, endometrioid and clear cell). We also determined that PAX8 is expressed in the non-ciliated, secretory cells of healthy fallopian tube mucosal linings but not in the adjacent ciliated epithelia.
Conclusion.

These findings support the hypothesis that PAX8 plays parallel roles in the development of epithelial ovarian cancer and in the developmental differentiation of coelomic epithelia into endosalpingeal epithelia.
INTRODUCTION

Epithelial ovarian carcinoma (EOC) is believed to arise from the single layer of cells found surrounding the ovary, referred to as ovarian surface epithelia (OSE). The OSE are the modified coelomic or peritoneal mesothelia that form a single layer of flat-to-cuboidal cells covering the ovary [1]. Interestingly and unlike most other epithelial derived cancers, the phenotypically uncommitted OSE undergo complex epithelial differentiation as they progress to form polarized epithelia, papillae, cysts and glandular structures, i.e., the cellular differentiation of EOC mimics the development of other differentiated epithelia of the female urogenital and abdominal regions [1].

Extensive microarray data have been generated to establish the gene expression profiles of ovarian carcinoma tissues and cell lines [2-19]. These studies produced numerous candidate molecular markers for the study and identification of epithelial ovarian cancer. The molecular mechanisms by which the altered expression of these genes contributes to the onset and progression of ovarian cancer are largely unknown.

Marquez et al. recently reported that when compared with normal ovarian epithelial brushings, alterations in microarray gene expression profiles of serous tumors correlated with those in normal fallopian tube (P = 0.0042) but not in other normal tissues [14]. This indicates that EOCs not only imitate the phenotype of other differentiated epithelium of the female reproductive tract, but their gene expression profiles as well. One gene consistently over expressed in EOC relative to normal ovarian tissue is paired box gene 8 (PAX8, X69699) [2, 10]. Mammalian paired box genes encode a family of 9 transcription factors (PAX1-PAX9) involved in embryogenesis [20]. PAX8 is thought to play a regulatory role in cell fate decisions during the development of the thyroid and
kidney [21]. Multiple splice variants of PAX8 have also been identified in Wilms’
tumors of the kidney [21]. PAX8 is known to have at least five different splice variants,
A-E [22]; however, the significance of these splice variants is presently unknown. By
cDNA microarray analysis, PAX8 was shown to be expressed at a significantly higher
level in ovarian cancer than in breast cancer [2]. In a subsequent oligonucleotide
microarray analysis, PAX8 was reported as one of the top 40 genes that was specifically
upregulated >2-fold in 20 primary serous papillary ovarian carcinomas and 17 ovarian
carcinomas metastatic to the omentum when compared to 50 normal ovaries and to over
300 other normal and diseased tissues [10]. However, characterization of PAX8
expression by other experimental methods has been limited [23]. In order to validate the
aforementioned microarray results and to further explore the role PAX8 may play in the
etiology of EOC, the expression of PAX8 was characterized in four subtypes of EOC
tissues, the EOC cell lines BG-1 and OVCAR-3, healthy ovaries and healthy fallopian
tubes.

RESULTS

Immunohistochemistry of PAX8 in ovarian tissue

To establish the presence and cellular localization of PAX8 protein in EOC tissue,
an immunohistochemical (IHC) screen of archived paraffin embedded samples was
performed. Tissues were assayed from 19 serous, 14 endometrioid, 18 mucinous, and
two clear cell subtypes of EOC. Non-neoplastic ovarian tissue from eight patients was
assayed as a negative control while thyroid tissue was used as a positive control for
PAX8 staining since PAX8 expression is required for proper function of the thyroxin-
producing follicular epithelia of the mammalian thyroid [24, 25]. Strong nuclear staining of PAX8 in the follicular cells of the thyroid gland was detected, (Figure 1A, B) while the OSE, stroma and granulosa cells lining normal follicles in the ovaries were negative for PAX8 immunoreactivity (Figure 1C, D). Interestingly, the epithelial inclusion cysts of healthy ovaries were positive for PAX8 expression (Figure 1E, F). In one instance of a cyst that had undergone tubal metaplasia, the PAX8 staining was notably intense (Figure 1F, arrowhead). IHC also demonstrated strong positive nuclear expression of PAX8 in the epithelia of ~70% of serous, endometrioid, and clear-cell subtypes of EOC. Representative images of positive PAX8 staining in serous, endometrioid and clear-cell subtypes of EOC are shown in Figure 1G-I. In contrast, mucinous subtypes of EOC display PAX8 expression in only ~10% of screened individuals (Figure 1J). A summary of the results of our IHC of ovarian tissue samples is shown in Table 1.

**PAX8 expression in fallopian tube secretory epithelium.**

While screening normal ovarian tissue for the presence of PAX8 by IHC, the presence of fallopian tube (oviduct) sections was detected in several samples. Intense PAX8 staining was detected in the epithelia that line the inner mucosa of the oviduct (Figure 2A). The endosalpingeal lining is composed of simple columnar epithelium consisting of two cell types, ciliated and secretory [26]. The ciliated cells aid in the capture of the ovulated egg for fertilization and the subsequent transport to the uterus. Secretory cells are functionally similar to goblet cells of the intestinal and respiratory tracts, providing lubrication as well as nutrition for the fertilized egg. Upon further
examination, it was determined that only the secretory subset of epithelia in the oviduct mucosa was positive for PAX8 expression. Higher magnification exposed complex patterns of ciliated cells separated by PAX8 expressing secretory cells (Figure 2B, C). An endosalpingeal phenotype was also seen in several of the simple inclusion cysts that stained positively for PAX8 in the healthy individuals. More detailed examination of these cysts revealed regions where the ciliated cells were also negative for PAX8 expression (Figure 2D, E).

Characterization of the PAX8 gene locus in EOC tissue and cell lines.

A translocation identified in a subset of human thyroid follicular carcinomas, t(2;3)(q13;p25), results in the fusion of the DNA binding domains of PAX8 to domains A to F of the peroxisome proliferator-activated receptor g1 (PPARg1) [27]. The resulting fusion protein (~97 kDa) has been hypothesized to play an oncogenic role in thyroid follicular carcinomas. To determine the molecular size of PAX8 present in our samples, immunoblots and RT-PCR were carried out on total protein and ribonucleic acid (RNA) isolated from two phenotypically divergent EOC cell lines (BG-1 [28] and OVCAR-3 [29], described further below), a section of the ampulla from a healthy fallopian tube and the tissue of three individual cases of serous papillary EOC. In the OVCAR-3 cell line, the fallopian tube and the three cases (CA183, -369, -413) of serous papillary ovarian carcinoma examined, the expected 48 kDa protein of the full-length PAX8A isoform was observed by immunoblot analysis (Figure 3A). Furthermore, RT-PCR of these same samples indicated the presence of multiple splice variants of PAX8 in these tissues (Figure 3B). Cloning and sequencing of the RT-PCR products from the OVCAR-3 cell
line revealed the presence of PAX8 isoforms A, B, C, and D (Figure 3C). The same four splice variants were also detected in cancer sample 183. In the samples with less abundant RT-PCR product, only isoforms A, B and C were observed. These results do not rule out the presence of isoform D at lower abundance in these tissues. In all samples, the PAX8A isoform was most abundant. Primers for RT-PCR were designed to span the three breakpoints in exons 8, 9 and 10 of PAX8 in the PAX8-PPARγ1 fusions identified previously [27] (Figure 3C). The results verify that these exons remain intact in EOC and demonstrate the presence of native PAX8 expression in these samples. These results also confirmed that the expression pattern of PAX8 in fallopian tube tissue is identical to that found in EOC.

BG-1 and OVCAR-3 are two independently established EOC cancer cell lines with distinct phenotypes in two-dimensional cell culture. BG-1 cells grow in isolation with elongated, mesenchymal features, whereas OVCAR-3 grows with the characteristic “cobblestone” phenotype of typical epithelial cells (Figure 4A, B). In addition, abundant intracellular vacuoles are found in the perinuclear and cytoplasmic regions of many OVCAR-3 cells (Figure 4B) but are lacking in BG-1 cells. Immunoblot analysis of these two cell lines revealed that PAX8 is highly expressed in OVCAR-3 cells but is absent in BG-1 cells when compared to endogenous beta actin (ACTB) (Figure 3A). Likewise, RT-PCR of total RNA from the two cell lines confirmed the presence of PAX8 in the OVCAR-3 cell line and its absence from BG-1 (Figure 3B). IHC using PAX8 specific antibodies incubated with BG-1 and OVCAR-3 cells revealed intense nuclear localization of PAX8 in the OVCAR-3 cell line, as expected for this transcription factor (Figure 4D). Nuclear staining was low or nonexistent for PAX8 when BG-1 cells were examined
(Figure 4C). These results establish OVCAR-3 as a viable cell culture model for investigating the role of PAX8 in EOC.

**DISCUSSION**

It is widely accepted that an understanding of developmental evolution is dependant upon knowledge of the “genetic architecture,” i.e. the number of genes and the relative effect of individual genes contributing to a particular cellular or organismal trait [30]. It is our contention that an understanding of the development and evolution of the individual types and subtypes of ovarian cancer is likewise dependent on knowledge of their genetic architecture. While the inappropriate expression of specific sets of the master-regulatory developmental homeobox (HOX) genes has been associated with the differentiation of individual subtypes of EOC, little else is known about the transcriptional regulation of EOC.

PAX proteins, which are defined by the presence of a sequence specific DNA-binding domain, the ‘paired box,’ are involved in organogenesis during mammalian development [20]. PAX8 is specifically expressed in the developing mammalian thyroid and kidney [21]. In the developing kidney, PAX8 expression is localized to the differentiating regions that undergo a mesenchymal to epithelial transition (MET) [22]. Likewise, the morphologic changes that OSE undergo as they form cysts inside the ovary may also be considered an instance of MET. The OSE possess mesenchymal markers such as N-cadherin, whereas inclusion cysts express the epithelial marker E-cadherin [1]. Interestingly, the condensing mesenchyme of the developing kidney, the OSE, and the oviduct epithelia are all derived from a portion of the intermediate mesoderm, known as
the urogenital mesenchyme, that gives rise to both the mammalian kidneys and gonads [31]. Considering their developmental origin, OSE may be primed to execute the MET that is thought to coincide with the expression of PAX8 in other tissues. Our demonstration that PAX8 is expressed in the EOC cell line, OVCAR-3, which displays an epithelial phenotype, is consistent with this hypothesis.

While one instance of PAX8 being used as a marker of Mullerian duct development in mouse has been reported [32], the data presented here constitute the first evidence that PAX8 expression is limited to the non-ciliated, secretory or peg cells of the human adult fallopian tube. This unique expression pattern of PAX8 in the human oviduct may shed further light on the regulation of PAX8 during normal human development as well as its aberrant expression in EOC. Consistent with this notion, the serous subtype of EOC and ovarian inclusion cysts are reported to resemble the secretory epithelia found on the inner lining of the fallopian tube [33].

Our results indicate that benign ovarian inclusion cysts display a pattern of ciliated and non-ciliated cells with PAX8 presence being limited to the latter. The non-malignant development of ciliated cells in the oviducts and ovarian cysts of mammals likely represents a terminally differentiated state of these epithelial cells. Interestingly, ciliated cells are not seen in malignant carcinomas of the ovary. Our results suggest that the lack of ciliated cells in malignant carcinomas of the ovary may be the result of deregulation of a normal developmental pathway of oviduct epithelia. The differential expression of PAX8 between ciliated and non-ciliated epithelium of the oviduct, ovarian cysts and EOC further suggests that PAX8 may play a regulatory role in execution of the serous/secretory phenotype of the cells from these tissues.
Oviduct epithelia are known to be important for creating the environment necessary for successful mammalian oocyte fertilization (reviewed in [34]); however little is known about the genes that control their embryonic development and continued cyclic differentiation in mammalian females. In the baboon, the morphological and functional state of the oviductal epithelium has been observed to be under the control of the ovarian steroids estradiol and progesterone [35]. In a study on prepubertal beagles, it was shown that in response to estradiol, low cuboidal cells of the oviduct underwent hypertrophy, hyperplasia, and cytodifferentiation to give rise to columnar ciliated and secretory cells [36]. However, if estradiol treatment was terminated or progesterone was given alone or in conjunction with estradiol, the oviductal epithelium regressed to low cuboidal cells with only 25% of the cells possessing cilia. Similar findings have been reported in the cat, with the added observation of apoptosis occurring in oviduct epithelium in animals chronically treated with progesterone and estradiol [37]. These observations suggest a continually regulated cycle of growth, differentiation, death and renewal in the epithelium of the mammalian oviduct. Therefore, an integral role in the development of endosalpingeal epithelium may be the ability of PAX8 to promote cell survival. In support of this hypothesis, it has been shown that PAX2, 5 and 8 are capable of repressing the transcription of tumor protein p53 (TP53) [38]. TP53 is a direct transcriptional activator of the pro-apoptotic gene, BAX [39]. Therefore, transcriptional repression of TP53 by PAX2, 5 or 8 may lead to the down regulation of BAX followed by the suppression of apoptosis [38]. However, the regulation of TP53 by PAX2, 5 or 8 may prove to be tissue specific, for it has been shown that the presence or absence of PAX5 has no effect on the transcription of TP53 in B-lymphocytes [40].
The proto-oncogene, *B-cell CLL/lymphoma 2 (BCL2)*, suppresses apoptosis in a number of cell types and is essential for mammalian kidney development [41]. It has been noted that BCL2 and PAX8 demonstrate a nearly identical pattern of expression in the mammalian kidney [42]. In addition, PAX8 has been shown to transcriptionally activate *BCL2* expression, which may lead to the subsequent suppression of apoptosis and the consequent development and maintenance of renal tubular epithelial structures [42]. BCL2 is also highly expressed in the cytoplasm of secretory epithelia of the fallopian tube; whereas ciliated cells are completely negative [43]. As we have shown that PAX8 is expressed in this same cell-specific fashion, PAX8 may regulate *BCL2* expression in the secretory cells of the fallopian tube as well. Mediation of anti-apoptotic signals via PAX8 expression would offer a selective advantage to OSE that become dislodged from their basement membrane on the outer surface of the ovary and subsequently migrate into the cortex of the ovary to form epithelial inclusion cysts.

Recently, the study of prophylactic salpingo-oophorectomies from women with BRCA mutations has identified the fallopian tube as a recurrent site of origin for serous carcinoma in these individuals [44]. This has led some to suggest that the tubal epithelium may also be the cell of origin for many sporadic ovarian serous carcinomas [45]. This is in contrast with the more widely accepted, albeit controversial, OSE origin of sporadic ovarian serous carcinomas. Our finding that PAX8 is expressed in tubal epithelia and EOC supports other data indicating these tissues not only resemble each other at the histopathological level but also at the gene expression level [14]. Additional data will be needed to address the role of the fallopian tube in the origin of sporadic ovarian serous carcinomas.
In summary, our results suggest that PAX8 not only plays a role in the uncontrolled growth of EOC but also in the regulated development of endosalpingeal epithelia. We are currently investigating the role of PAX8 in executing these two, not mutually exclusive phenomena.

Materials and Methods

Human Tissues
Archival formalin-fixed, paraffin embedded tissue from ovarian carcinoma of different histological subtypes, non-neoplastic ovarian, fallopian tube and thyroid tissues were obtained from the files of Emory University hospital and Crawford Long hospital, Atlanta, GA. The PAX8 immunohistochemistry screen of these tissues was approved by the Emory University's Institutional Review Board (IRB). Fresh EOC and fallopian tube tissue for protein and RNA isolation was provided by the Ovarian Cancer Institute (OCI), Atlanta, GA. Patients were enrolled in the OCI study under a protocol approved by the Northside Hospital IRB, Atlanta, GA.

Immunohistochemistry
Sections of formalin-fixed, paraffin-embedded tissue (5 microns) were deparaffinized and rehydrated. Antigen retrieval was in citrate buffer (pH 6) using an electric pressure...
cooker for 5 minutes at 120 C with cooling for 10 minutes before immunostaining. All tissues were exposed to 3% hydrogen peroxide for 5 minutes, primary antibody (α-mPax8-bIII, 1:200 dilution) for 30 minutes, DAKO ENVISION system (DAKO Corp) HRP labeled polymer conjugated with secondary antibody for 30 minutes, diaminobenzidine as chromogen for 5 minutes, and DAKO automated (DAKO AUTOSTAINER) hematoxylin and counterstain for 15 minutes. All incubations were performed at room temperature. Between incubations, sections were washed with tris-buffered saline (TBS) buffer. Coverslipping was performed using the Tissue-Tek SCA (Sakura Finetek USA, Inc.) automatic coverslipper. Slides were scored by a board certified pathologist on a scale of zero to 3+. Briefly, the immunohistochemical expression of PAX8 was semi-quantitatively scored as 1+ (11-40% of tumor cells with positive staining); 2+ (41-70% of tumor cells with positive staining) and 3+ (greater than 71% of tumor cells with positive staining). Tumors with less than 10% of cells staining were considered as negative. The intensity of staining was highly variable between tumors as well as within foci in a single tumor slide and hence only percentage positive expression was recorded for analytical purposes. Slides were photographed with an Olympus C5050 digital camera attached to the optical port of an Olympus BX60 compound microscope.

BG-1 and OVCAR-3 cells were stained for PAX8 using the Vector Elite ABC Peroxidase system (DAB) (Vector Laboratories, Burlingame, CA). All incubations were performed at room temperature, and slides were washed twice for 5 min with 1X PBS between all incubations. Cells (1x10^5-2x10^5) were plated onto glass Lab-Tek® chamber slides (Nalge Nunc International, Rochester, New York) and incubated overnight at 37°C.
in a 5% humidified CO$_2$ atmosphere. Cells were fixed by incubating the slides in methanol for 30 min followed by a 15 min incubation using 0.3% H$_2$O$_2$ in methanol to block endogenous peroxidase activity. Slides were then incubated in a 1% gelatin/PBS (Mallinckrodt Baker, Inc., Phillipsburg, NJ) blocking solution for 20 min at room temperature. Excess gelatin/PBS was removed and the slides incubated in a humid chamber with a polyclonal antibody against PAX8 ($\alpha$-mPax8-bIII, 1:1000) for 1 h at room temperature followed by incubation with a biotinylated goat anti-rabbit secondary in 1% BSA/PBS for 30 min. The ABC-peroxidase complex was prepared according to the manufacturer’s instructions and the slides incubated with the complex for 1 h followed by staining with 3,3’-diaminobenzidine tetrahydrochloride (DAB) for 2-5 min. Cells were then counterstained with hematoxylin and coverslipped.

**Immunoblotting**

Proteins were isolated from fresh, snap-frozen EOC tissue by homogenization in CellLytic MT Mammalian Tissue Lysis/Extraction Reagent (Sigma). The homogenate was then diluted with 2X Laemmli protein loading buffer (0.5M Tris-HCl (pH 6.8), 4.4% (w/v) SDS, 20% (v/v) glycerol, 2% (v/v) 2-mercaptoethanol, and bromophenol blue in distilled/deionized water), heated to 95°C for 5 minutes and the supernatants cleared by microcentrifugation. The BG-1 and OVCAR-3 cell lines were grown to a density of ~ 5 X 10$^6$ cells/10cm plate and lysed with 2X Laemmli loading buffer (SDS, Bromophenol Blue) directly in the culture flasks. Homogenates were heated to 95°C for 5 minutes and subjected to microcentrifugation. After separation by 10% SDS-PAGE, proteins were
electroblotted on to Immuno-Blot PVDF membrane (Bio-Rad Laboratories, Hercules, CA) at a constant voltage of 60V for 2 hours. The membrane was blocked with 8% nonfat dry milk in 1X phosphate buffered saline (PBS) by rocking for 1 hr at room temperature. Immunodetection was performed using a specific anti-PAX8A rabbit polyclonal antibody (α-mPax8-bIII, kindly provided by Dr. Roberto Di Lauro, Stazione Zoologica, Napoli, Italy) diluted 1:1000 in 0.03% Tween-20/1X PBS solution and rocked overnight at 4°C. A goat anti-rabbit IgG, peroxidase conjugated secondary antibody (AP307P, Chemicon, Temecula, Ca) diluted 1:10000 in 0.03% Tween-20/1X PBS and rocked for 1 hour at room temperature was used for chemiluminescent detection. Between incubations, membranes were washed three times for 5 minutes in 0.3% Tween-20/1X PBS. The membranes were developed using SuperSignal West Pico reagent (Pierce Biotechnology, Rockford, Illinois) according to the manufacturer’s protocol. Immediately following detection, the membrane was stripped for 10 minutes with gentle agitation at room temperature in 10 ml of Restore Western Blot Stripping Buffer (21059, Pierce Biotechnology). Immunodetection for beta actin (ACTB) was performed using a 1:5000 dilution of a mouse monoclonal antibody (MAB1501, Chemicon) and a 1:10000 dilution of a goat anti-mouse IgG, peroxidase conjugated secondary antibody (AP308P, Chemicon) as described above.

RT-PCR and Sequencing

Total RNA was isolated from the EOC cell lines and fresh tissue stored in RNA later using TRIzol Reagent (Invitrogen Corporation, Carlsbad, CA) following manufacturer’s
protocols. cDNA was synthesized from 300 ng of total RNA with the SuperScript III System (Invitrogen) using random hexamers. PCR was performed using one primer located in exon 5 of \( PAX8A \) (NM_003466) with the sequence 5’-TCAACCTCCCTATGGACAGC-3’ and one primer in exon 11 with the sequence 5’-GCCTCGCTGTAGGAGGAGTA-3’. These primers were chosen to span the reported breakpoints of \( PAX8 \) in follicular thyroid carcinomas [27]. RT-PCR products from the OVCAR-3 cell line were cloned into the pCR2.1-TOPO plasmid (Invitrogen). Plasmid DNA was isolated with the QIAprep Spin Miniprep Kit (Qiagen Sciences, Maryland, USA) and screened for insert size by restriction digestion with EcoRI (New England BioLabs, Ipswich, MA) and gel electrophoresis before sequencing at the School of Biology Genome Center at the Georgia Institute of Technology (http://www.biology.gatech.edu/genomecenter/).

Cell Culture

The BG-1 cell line was kindly provided by Julie M. Hall and Kenneth S. Korach, Receptor Biology Section, Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, NIH, Division of Intramural Research, Environmental Disease and Medicine Program, Research Triangle Park, NC. BG-1 cells were propagated in DMEM:F12/50:50 (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and penicillin and streptomycin (Mediatech, Inc., Herndon, VA) at 37°C, 5% CO\(_2\) and ~80% relative humidity. The OVCAR-3 cell line was purchased from American Type Culture Collection, Manassas, VA (ATCC number: HTB-161). OVCAR-3 cells were propagated in RPMI 1640 medium (Invitrogen)
adjusted to contain 2 mM L-glutamine, 10 mM HEPES, and 1.0 mM sodium pyruvate and supplemented with 0.01 mg/ml bovine insulin (Sigma, St. Louis, MO), 20% fetal bovine serum (Invitrogen) and penicillin and streptomycin.

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Competing interests. The authors have declared that no competing interests exist.

Abbreviations
ACTB (beta act), BCL2 (B-cell CLL/lymphoma 2), EOC (Epithelial Ovarian Carcinomas), IHC (Immunohistochemical, Immunohistochemistry), MET (Mesenchymal to Epithelial Transition), OSE (Ovarian Surface Epithelia), PAX8 (paired box gene 8), TP53 (tumor protein p53)

References


Figure Legends

**Figure 1.** PAX8 Immunolocalization in Epithelial Ovarian Cancer Tissue

(A) and (B) PAX8 immunostaining of healthy thyroid tissue indicating strong nuclear staining in the follicular epithelia, 200X (A) and 400X (B).

(C) and (D) PAX8 immunostaining of healthy ovarian tissue indicating that the ovarian surface epithelium (arrow), stroma (asterisk) (C) and follicular cells (D) are negative for PAX8 staining (200X).

(E) and (F) PAX8 immunostaining of healthy ovarian tissue indicating positive staining for epithelial inclusion cysts. Arrow indicates a cyst that has undergone tubal metaplasia.

(G) and (H) and (I) Representative images of PAX8 immunostaining of serous (G), endometrioid (H) and clear cell (I) subtypes of epithelial ovarian cancer tissues indicating strong nuclear staining in the cancer epithelium.

(J) Representative image of PAX8 immunostaining of a mucinous subtype of epithelial ovarian cancer indicating no staining.

**Figure 2.** Immunolocalization of PAX8 to the Non-ciliated, Secretory Epithelium of Healthy Fallopian Tubes and Ovarian Epithelial Inclusion Cysts.

(A), (B) and (C) PAX8 immunostaining of healthy fallopian tubes indicating staining of the mucosa (A), 400X. In (B) the arrow indicates that only the non-ciliated, secretory epithelia or PEG cells stain positive for PAX8, 600X. In (C), a pattern of PAX8 positive intercalating cells are separated by ciliated (indicated by arrow) PAX8 negative cells, 1000X.
(D) and (E) Immunostaining of an epithelial inclusion cyst from a healthy ovary indicates a similar pattern of PAX8 immunoreactivity in non-ciliated cells, 600X (D) and 1000X (E). The arrow in (E) indicates the presence of PAX8-negative ciliated cells in the cyst.

**Figure 3.** PAX8 Immunoblotting and RT-PCR of Epithelial Ovarian Cell Lines, Fallopian Tube Tissue and Epithelial Ovarian Cancer Tissue

(A) Immunoblot analysis for PAX8 in the BG-1 and OVCAR-3 cell lines, fallopian tube tissue and three individual cases of epithelial ovarian cancer indicating the absence of PAX8 from the BG-1 cell line and presence of the expected 48 kDa protein in the OVCAR-3 cell line, fallopian tube tissue and the three cases of ovarian serous papillary adenocarcinoma. Immunoblot detection of ACTB is shown as a loading control

(B) RT-PCR of the same samples as in A indicating the presence of PAX8 splice variants A, B, C and D found in the samples. BG-1 showed no expression of PAX8. A negative control of the OVCAR-3 cDNA reaction minus RT is indicated. RT-PCR for ACTB is shown as a positive control for each sample.

(C) Schematic indicating the exon/intron structure of PAX8A, -B, -C and -D and the location of the primers used for RT-PCR in (B).

**Figure 4.** PAX8 Immunolocalization in Epithelial Ovarian Cancer Cell Lines

(A) Image of BG-1 cell line growing in two-dimensional culture. Notice the fibroblastic, elongated, mesenchymal cellular phenotype.

(B) Image of OVCAR-3 cell line growing in two-dimensional culture. Notice the “cobblestone” epithelial phenotype.
(C) PAX8 immunostaining of BG-1 cell line indicating no staining.

(D) PAX8 immunostaining of OVCAR-3 cell line indicating abundant nuclear staining.
**Table 1.** PAX8 Immunohistochemical Expression in Subtypes of Epithelial Ovarian Carcinoma.

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<tr>
<th>Histologic type</th>
<th>% Positive</th>
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<tr>
<td>Serous</td>
<td>79% (15/19)</td>
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<tr>
<td>Endometrioid</td>
<td>64% (9/14)</td>
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<tr>
<td>Clear cell</td>
<td>100% (2/2)</td>
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<tr>
<td>Mucinous</td>
<td>11% (2/18)</td>
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