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"ROLE OF PAX8 IN THE TUMORIGENESIS OF OVARIAN CANCER"

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ABSTRACT

PAX8 is a member of the Paired box (PAX) multigene family of transcription factors, which are involved in the tissue-specific control of the expression of several genes during development of both vertebrates and invertebrates. Previously, PAX8 has been studied as key molecular marker and regulator of follicular thyrocyte differentiation, but recent evidence show that PAX8 is also expressed in specific types of tumors. In particular, PAX8 results to be expressed in human ovarian cancer subtypes and Fallopian tubal secretory cells from which the ovarian cancer may originate. However, the functional role of PAX8 in the carcinogenesis of ovarian cancer has not been addressed yet.

In this study, we investigated the potential role of PAX8 in ovarian cancer progression, using *in vitro* ovarian cancer cells and *in vivo* mouse xenograft models. To this purpose, stable PAX8 depleted ovarian cancer cells (SKOV-3) were generated using short hairpin RNA (shRNA) constructs. Cell proliferation, motility and invasion potential of PAX8 silenced cells were analyzed by means of growth curves, wound healing and matrigel assays. Furthermore, PAX8 knockdown and control cells were injected into nude mice for xenograft tumorigenicity assay. The results obtained *in vitro* showed that PAX8 is involved in the regulation of proliferation, migration and invasion of ovarian cancer cells. In addition, PAX8 silencing strongly suppresses cellular anchorage-independent growth *in vitro* and, notably, tumorigenesis *in vivo* in the nude xenograft mouse model. Overall, these results indicate that PAX8 plays an important role in the tumorigenic phenotype of ovarian cancer cells.

Finally, to identify new genes and pathways modulated by PAX8 in ovarian cancer, an expression profile analysis was performed by RNA sequencing (RNA-seq) of PAX8 knockdown ovarian cancer cell (SKOV-3) and Fallopian tube cells (FT-194). Twenty-four hours after PAX8 knockdown in SKOV-3 and FT-194, 182 and 164 genes were found to be modulated, respectively. These genes resulted to be correlated to different biological pathways involved in cancer progression. This is the first RNA-seq study that compares genes regulated by PAX8 in ovarian cancer cells and Fallopian tube cells. The identification of the biological pathways and target genes controlled by PAX8 will have considerable importance to understand ovarian cancer progression as well as to set up novel therapeutic strategies.

1. BACKGROUND

1.1 Ovarian cancer: incidence, pathogenesis and classification

Worldwide, approximately 200.000 women are diagnosed with ovarian cancer annually, with an estimated 100.000 associated deaths (Jemal et al. 2011). Although ovarian cancer accounts for only 3% of all cancers in women, it has one of the highest death-to-incidence ratios, which has been primarily attributed to the unavailability of effective screening tools, to the absence of early phase symptomatology in many patients, and to its typical presentation at advanced stages when the prognosis is poor (Jemal et al. 2008). One of the greatest obstacles to the detection of early-stage ovarian cancer is our poor understanding of its histogenesis and pathogenesis.

The origins of ovarian cancer are complex and still under debate. Several models have been posited to explain how epidemiological factors such as menstruation and ovulation may lead to ovarian cancer (Figure 1).

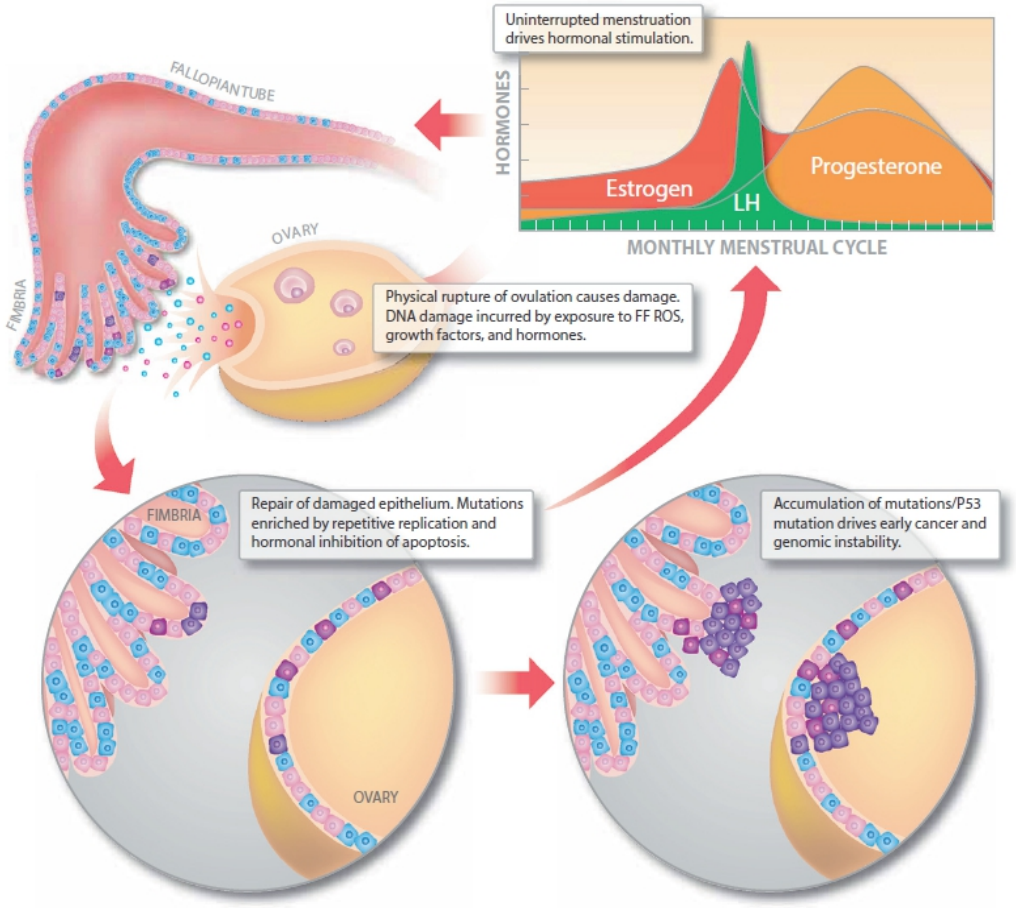

Figure 1. ***Incessant Ovulation hypothesis and Gonadotropin hypothesis for ovarian cancer pathogenesis*** *(from Emori et al., 2014)*

A long standing hypothesis, called the Incessant Ovulation hypothesis, suggests that the repetitive wounding and healing of the ovarian surface epithelium and adjacent tubal epithelium, that is induced by monthly ovulation, increases cell proliferation and thus the likelihood of genomic instability which could lead to oncogenesis (Emori and Drapkin 2014). Another hypothesis, known as the Gonadotropin hypothesis, implicates excessive direct and indirect stimulation of the ovarian surface epithelium by gonadotropins, leading to differentiation, proliferation, and ultimately malignant transformation (Cramer and Welch 1983). More recently, the Incessant Menstruation hypothesis suggests that repeated retrograde menstruation exposes the ovary and Fallopian tubes to the genotoxic effects of reactive oxygen species and iron-associated oxidative stress (Vercellini et al. 2011). Lastly, several recent papers have focused on damage induced by inflammation-mediated factors found in the follicular fluid (Backman et al. 2014, Lau et al. 2014). The recurring theme in all these hypotheses is the incessant ovulatory damage that reinforces the importance of ovulation in ovarian cancer progression, but also makes it difficult to separate the impact of each hypothesis as they are physiologically interconnected. Women with altered steroid hormone levels, such as those with Polycystic Ovarian Syndrome, tend to ovulate sporadically and to rarely develop ovarian cancer as well as women who take oral contraceptives and receive the protective benefits of both lowered gonadotropin levels and inhibited ovulation (McCartney et al. 2002). However, the average age of ovarian cancer onset is postmenopausal, at age 63, when hormonal levels have shifted and ovulation has stopped (Emori and Drapkin 2014). Thus, an unanswered question that also remains is why menopause is so temporally important to the onset of the disease.

Based on morphologic, immunohistochemical and molecular data, a dualistic model of ovarian carcinogenesis has been proposed that classifies ovarian carcinomas into 2 groups: type I and type II. (Figure 2)

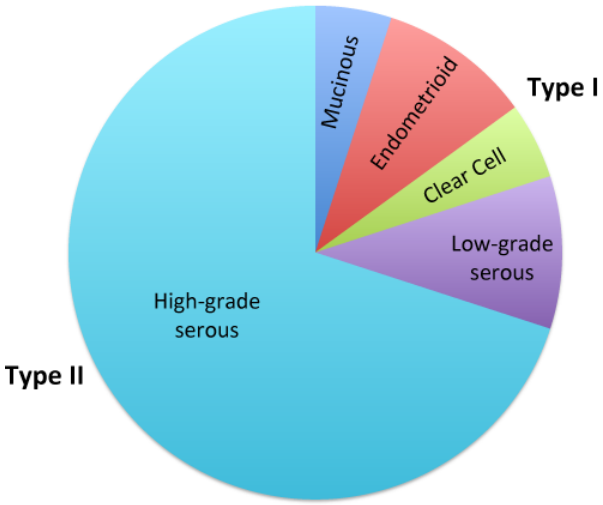

Figure 2. ***Classification of ovarian carcinomas into type I and type II.***

Type I tumors include low-grade serous carcinomas (LG-SC), low-grade endometrioid carcinomas, clear cell and mucinous carcinomas that are not clinically aggressive and generally present at early stage. They rarely harbor TP53 mutations, but instead display mutations involving specific cell signalling pathways, including KRAS, BRAF, ERBB2, PTEN, CTNNB1, PIK3CA, ARID1A, and PPP2R1A. Women with LG-SC had prolonged median survival of 81 months compared to 57–65 months in those with high-grade epithelial ovarian cancer (Ozlos et al. 2003, Gershenson et al. 2006, Armstrong et al. 2006). Pathologic findings show that 60% of LG-SC are associated with a low malignant potential (LMP) neoplasm, whereas it was found in only 2% of high-grade ovarian carcinomas. Moreover, recurrent serous ovarian tumors with LMP are mostly low-grade lesions and have slow progression (Crispens et al. 2002, Malpica et al. 2004).

Type II tumors, which include high-grade serous carcinomas (HGSC), high-grade endometrioid carcinomas, malignant mixed mesodermal tumors (carcinosarcomas), and undifferentiated carcinomas, frequently display TP53 mutations and are genetically unstable (Kurman and Shih 2011). High-grade malignancies tend to be fast growing and chemosensitive, and the low-grade neoplasms typically grow slowly, but are less sensitive to chemotherapy (Shih and Kurman 2004). Lifetime ovulation is positively correlated with HGSC, the most common type II tumor, and factors, such as birth control which decreases lifetime ovulation, have a protective effect against HGSC (Cramer and Welch 1983). Thus, establishing the molecular mechanisms linking ovulation to HGSC pathogenesis could be critical to develop new screening techniques and treatments for the disease.

1.2 Genetic and protein alteration in ovarian cancer

Genetic and protein alterations in tumor cells also support the idea that the different types of ovarian cancers have unique pathogenesis (Table 1).

Characteristic	Low-grade	High-grade
TP53 inactivity	Rare	50-80%
HLA-G	Rare	61%
HER2/neu amplification	Rare	20-66%
AKT amplification	Rare	12-30%
Apoliprotein E expression	12%	66%
BRAF mutation	30-50%	Rare
KRAS mutation	30-50%	Rare
PTEN mutation	20% (endometrioid)	Rare
MSI	50% (endometrioid)	8-28%
ARID1A mutation	30% (endometrioid)	Rare

Table 1. ***Variability in biology of low- and high-grade tumors*** *(modified from Saad et al., 2010)*

Gene analyses have shown that BRAF, KRAS, and PTEN mutations occur more often in low-grade tumors compared to high-grade tumors (30–

50% versus 20%, respectively) (Singer et al. 2003a, Shih and Kurman 2004). Conversely, HLA-G, HER2, and AKT levels are increased in high-grade tumors (61%, 20–66%, and 12–30%, respectively) as compared to low-grade tumors (Singer et al. 2003b). While TP53 is found to be mutated in >80% of high-grade tumors, it is rarely mutated in low-grade tumors (Brachova et al. 2013).

As described in Table 2, several other oncogenes have been found deregulated in epithelial ovarian cancer (EOC).

Protein	Function	Rate in EOC
EGFR (HER-1)	Membrane TK receptor, promotes growth	35–70%
HER-2	Membrane TK receptor, promotes growth	20–66% (HGS)
Src	TK, promotes growth, angiogenesis, survival	80–90%
CSF-1/fms	Ligand/receptor, inhibits anoikis	50–70%
ILGF/ILGFR	Peptide hormone/receptor, promotes growth	21–25%
KRAS	G-protein, promotes growth through MAP kinase pathway	30–50% (LGS)
BRAF	Promotes growth through MAP kinase pathway	30–50% (LGS)
TGF-β	Ligand, inhibits growth through Rb activation	Lost in 40%
Myc	Transcription factor, cell cycle mediator	30%
Cyclin D/Cdk4/6	Advance from G1 to S phase	30–90%
Cyclin E/Cdk2	Advance from G1 to S phase	30–70%
Cyclin B/Cdk1	Advance cell cycle into M phase	80%
p16	Inhibits cyclin D/Cdk4/6	Lost in 30%
p27 (kip-1)	Inhibits cyclin E/Cdk2	Lost in 55%
p21 (WAF-1)	Inhibits cyclin B/Cdk1	Lost in 25–40%
PIP3/Akt	Akt (activated by PIP3) inhibits apoptosis	12–18% (HGS)
PTEN	Decrease Akt	20% (Endo)
p53	Promotes cell cycle arrest/apoptosis with DNA damage	50–90% (HGS)
BRCA1	Co-factor for transcription factors, “caretaker” of genome	6–82%a
BRCA2	Co-factor for transcription factors, “caretaker” of genome	1–3%
MLH1/MSH2	Mediates mismatch repair, promotes genetic stability	30% (Endo)
Fas ligand	Produced by tumor cells to induce apoptosis of T-cells	50–80%
HLA-G	Secreted by tumor cells to inhibit cytotoxic immune cells	61% (HGS)
hTERT	Subunit of telomerase, maintains telomere length	80–85%
VEGF/VEGFR	Ligand/receptor complex induces angiogenesis	40–100%
EphA2	TK promotes angiogenesis and vasculogenic mimicry	76%
MMPs	Matrix metalloproteinases degrade extracellular matrix	40–100%
αvβ3	Integrin, promotes survival and angiogenesis	95%
FAK	Co-factor TK promotes adhesion, proliferation, survival	70%
E-cadherin	Promotes adhesion	90–100%

Table 2. ***Main contributors to ovarian carcinogenesis*** *(modified from Saad et al., 2010)*

The tyrosine kinase Src plays a functional role in cell proliferation, adhesion, angiogenesis, and cell survival (Ishizawar and Parsons 2004, Silva 2004, Han et al. 2006), and in chemotherapeutic drug resistance (Pengetnze et al. 2003). Src over-expression is found in 93% of late-stage ovarian cancers and in more than 80% of ovarian cancer cell lines (Wiener et al. 2003). Inhibiting Src proto-oncogene results in the diminished growth of ovarian cancer in mouse models through inhibition of angiogenesis (Han et al. 2006).

Another group of proteins that result to be altered in ovarian tumorigenesis includes the type I tyrosine kinase receptor family HER (Erb) that comprises four monomers: EGFR (epidermal growth factor receptor, HER-1), HER-2 (proto-oncogene neu), HER-3, and HER-4. EGFR is over-expressed in 35– 70% of epithelial ovarian cancers (Bartlett et al. 1996), while HER-2 is over-expressed in 20–30% of ovarian cancer cases (Leary et al. 1992).

The oncoprotein RAS is a G-protein involved in cell proliferation. KRAS mutations have been detected in 61% of borderline tumors, 68% of low-grade tumors, 50% of mucinous adenocarcinomas, and only in 5% of high-grade serous carcinomas (Suzuki et al 2000, Singer et al. 2003a). However, only 19% of the LG-SC shows KRAS mutations (Wong et al. 2010). Activating mutations in BRAF have also been identified in ovarian tumors. An extensive study on BRAF and KRAS mutations performed in 264 epithelial and non-epithelial ovarian neoplasms showed that BRAF mutations occur exclusively in LG-SC (33 of 91, 36%) whereas KRAS mutations were identified in 26 of 91 (29.5%) LG-SC, 7 of 49 (12%) high-grade serous carcinomas, 2 of 6 mucinous adenomas, 22 of 28 mucinous borderline tumors, and 10 of 18 mucinous carcinomas. Of note, two serous borderline tumors were found to harbor both BRAF and KRAS mutations (Sieben et al. 2004).

At least 10% of all epithelial ovarian cancers are hereditary, with mutations in the BRCA genes accounting for approximately 90% of cases (Swisher 2003, Coupier et al. 2004, Prat et al. 2005). Hereditary ovarian cancers exhibit distinct clinicopathologic features compared with sporadic cancers. The cumulative lifetime risk of ovarian cancer is 40% to 50% for BRCA1 mutation carriers and 20% to 30% for BRCA2 mutation carriers. Both BRCA proteins participate in the transcriptional regulation of gene expression as well as the recognition or repair of certain forms of DNA damage, particularly double-strand breaks. Frame-shift or non-sense mutations of BRCA1 and BRCA2 are the most common. Most ovarian cancers associated with germline BRCA mutations are diagnosed at a younger age and are high-grade and advanced-stage serous carcinomas. BRCA mutations do not seem to play a significant role in the development of mucinous or borderline ovarian tumors. Hereditary ovarian cancers have a distinctly better clinical outcome with longer overall survival and recurrence-free interval after chemotherapy than sporadic cancers (Prat et al. 2005).

1.2 The Fallopian tubes as origin site for high-grade serious ovarian carcinoma

Ovarian carcinoma was traditionally thought to originate from the ovarian surface epithelium (OSE) or ovarian epithelial inclusions (OEI), and investigative efforts at early detection have accordingly been centered on the ovary for decades. However, these efforts have not been successful, as evidenced by the fact that the overall survival for women with ovarian cancer has not changed in any fundamental manner over the last 50 years. Several emerging lines of evidence indicate that some traditional knowledge of ovarian epithelial carcinogenesis and cellular origination are fundamentally incorrect.

Recently, studies on both asymptomatic women with germline BRCA1 or BRCA2 mutations as well as those from the general population with pelvic serous carcinoma, have detected precancerous or early cancerous lesions, serous tubal intraepithelial carcinoma (STIC), in the Fallopian tubal fimbria (Colgan et al. 2001, Powell et al. 2005, Medeiros et al. 2006). Furthermore, a spectrum of potential precursor lesions to serous carcinomas, including the 'p53 signatures' and the 'secretory cell outgrowth', have similarly been described in the Fallopian tube fimbria (Karst et al. 2014) (Figure 3).

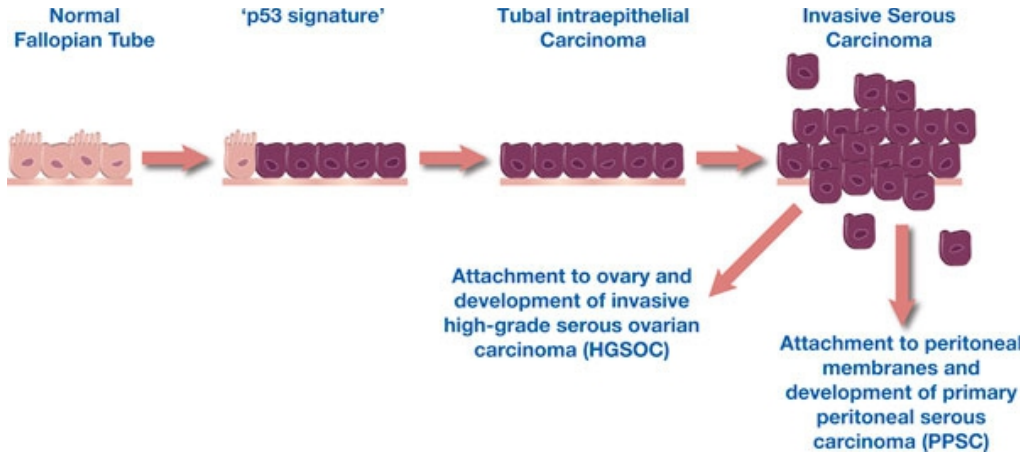

Figure 3. ***Illustration of the proposed carcinogenic sequence for serious tumorigenesis from the Fallopian tube epithelium*** *(from Karst et al., 2014)*

In addition, STIC lesions, which are defined as *in situ* cancers with TP53 mutations and increased proliferative capacity, are observed in at least 60% of women with HGSC of the ovary and/or peritoneum (Kindelberger et al. 2007; Przybycin et al. 2010) while similar *in situ* lesions are not observed in the OSE (Folkins et al. 2008). Such early lesions exhibit shortened telomeres, a notable hallmark of early molecular carcinogenesis (Kuhn et al. 2011, Chene et al. 2013). Clinical observations support the hypothesis that STICs can originate from secretory epithelial cells of the Fallopian tubes and progress to HGSC by rapidly disseminating to involve ovarian and peritoneal surfaces.

This hypothesis is further supported by the shared morphologic and immunophenotypic features of STICs and HGSCs. In addition, injection of transformed primary human Fallopian tube secretory epithelial cells (FTSECs) into the peritoneum of nude mice induces tumors that from histological, immunophenotypical and genomic point of view resemble human HGSCs (Karst et al. 2011, Jazaeri et al. 2011). However, these associations are largely circumstantial and necessitate experimental proof in order to confirm the role of FTSECs and STICs in serous carcinogenesis. From those and other studies, the Fallopian tube has emerged as an important potential source for female pelvic serous carcinomas, resulting in a paradigm shift that will likely have important implications for future detection, therapy and prevention in ovarian cancer. Thus, these new theories furthermore suggest that different ovarian tumor subtypes have different origins, with the ovarian surface epithelium implicated in type 1 tumors and Fallopian tube secretory epithelial cells implicated in HGSC (Levanon et al. 2008, Kurman and Shih 2010, Karst and Drapkin 2010).

Recently, it has been demonstrated that HGSC originates from the Fallopian tubal secretory epithelial cells that are positive for PAX8 expression (Perets et al. 2013). The identification of specific markers of the precursor Müllerian duct cells from the coelomic epithelium within normal endometrial and tubal mucosa provides strong evidence that supports the new carcinogenic hypothesis. These markers maintain their expression not only in endometrial or tubal tumors, but also in certain types of ovarian and peritoneal tumors. Within this context falls PAX8 protein that serves as an important marker for discriminating ovarian carcinomas from breast carcinomas, with greater sensitivity and specificity than Wilms tumor protein and it is particularly useful for the diagnosis of clear cell and endometrioid types of ovarian cancer. Although gene expression profiling studies have indicated that the transcription factor PAX8 is a potential diagnostic marker for ovarian carcinoma (Hibbs et al. 2004), the molecular mechanism by which PAX8 is involved in the carcinogenesis of these tumors remains unclear and requires further studies.

1.4 The transcription factor PAX8

PAX8 is a member of the PAX gene family that encodes for DNA binding proteins involved in the regulation of the development of various tissues in different species. The PAX gene family consists of nine well-described transcription factors (PAX1-9) highly conserved in vertebrates, and all these proteins are characterized by the presence of a Paired box. This specific sequence encodes for the Paired domain, a very important domain for recognizing the target sequences on the DNA. The Paired domain is composed of 128 amino acids and it is subdivided in two fundamental sub-domains, one at N-terminus, the other at the C-terminus, called PAI and RED, respectively. Each of these domains contains a helix-turn-helix motif and both domains are connected through a linker region (Mansouri et al. 1998) (Figure 4).

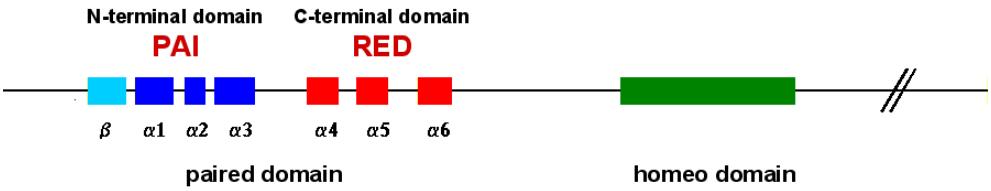

Figure 4. ***Schematic structure of a PAX protein***

The subdivision of the Paired domain into 2 independent domains reflects the ability of PAX proteins to bind DNA in different ways and, overall, to recognize different consensus sequences. Considering the homology of the Paired domains the nine PAX proteins can be subdivided in different groups (Figure 5).

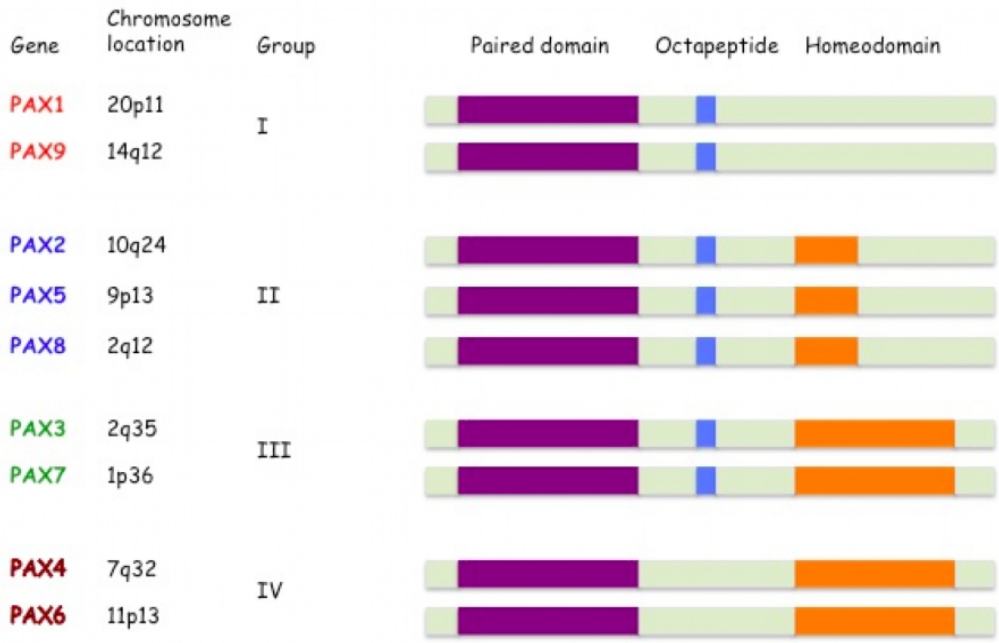

Figure 5. ***PAX family members subgroups***

The nine PAX proteins are divided into four subgroups based on the presence or absence of structural regions such as the DNA-binding homeodomain, the partial homeodomain and the octapeptide motif. All PAX genes contain the Paired domain and all contain an octapeptide region with the exception of PAX4 and PAX6. PAX2, 5 and 8 are characterized by the presence of an octapeptide and a partial homeodomain.

The temporal and spatial expression patterns of PAX genes are tightly regulated, and their expression is observed primarily during fetal development (Dahl et al. 1997). In most cases, PAX gene expression attenuates when development is complete, but in a few tissues it persists into adult life. Although abnormal cell growth and proliferation is often associated with high expression levels of PAX genes in adults (Muratovska et al. 2003), nevertheless, the precise role that PAX genes play in cancer is still unclear. During embryonic development, the protein PAX8 is expressed in thyroid, kidney, Muller system and nervous system (Pasca Di Magliano et al. 2000). It has been demonstrated to be required for both the morphogenesis of the thyroid gland and the maintenance of the thyroid differentiated phenotype. In addition to a key role in thyrocyte differentiation (Pasca Di Magliano et al. 2000), PAX8 is also required for the development of the Mullerian duct (Plachov et al. 1990). Indeed, PAX8 knockout mice have defects in the development of the Mulleran duct (Mittag et al. 2007), have a small thyroid, and show a severe hypothyroidism phenotype (Mansouri et al. 1998). Mutations of the PAX8 gene are among the genetic defects responsible for congenital hypothyroidism (De Felice and Di Lauro 2004).

In addition to its role in the pathogenesis of hypothyroidism, PAX8 also plays a role in the progression of follicular thyroid carcinomas and adenomas (Nonaka et al. 2008). It results to be overexpressed in the majority of gliomas, Wilms tumors and well-differentiated pancreatic neuroendocrine tumors (Poleev et al. 1992, Sangoi et al. 2011). Moreover, PAX8 is one of the top 40 genes specifically expressed in different types of ovarian carcinomas (Schaner et al. 2003). PAX8 is not expressed in the surface epithelial cells of the ovary, but its expression was found in 96% of serous ovarian carcinomas, in 89% of endometrioid and 100% of clear cell carcinomas (Nonaka et al. 2008). This observation could be justified by the fact that these tumors originate from Fallopian tubes cells where PAX8 is expressed.

1.5 PAX8 in Fallopian tubes and ovarian cancer

The transcription factor PAX8 is essential for the development of the female genital tract, including the Fallopian tubes but not the ovaries (Mittag et al. 2007). In fact, the female $PAX8^{-/-}$ mice are infertile because they lack a functional uterus revealing only remnants of myometrial tissue. In addition, the vaginal opening is absent. The infertility in $PAX8^{-/-}$ mice seems to be due to a defect in the development of the Mullerian duct rather than to hormonal imbalance, pointing to a direct morphogenic role for PAX8 in uterine development.

In the Fallopian tube epithelium (FTE), PAX8 is a marker of the secretory cell lineage, not of the ciliated cell population (Figure 6).

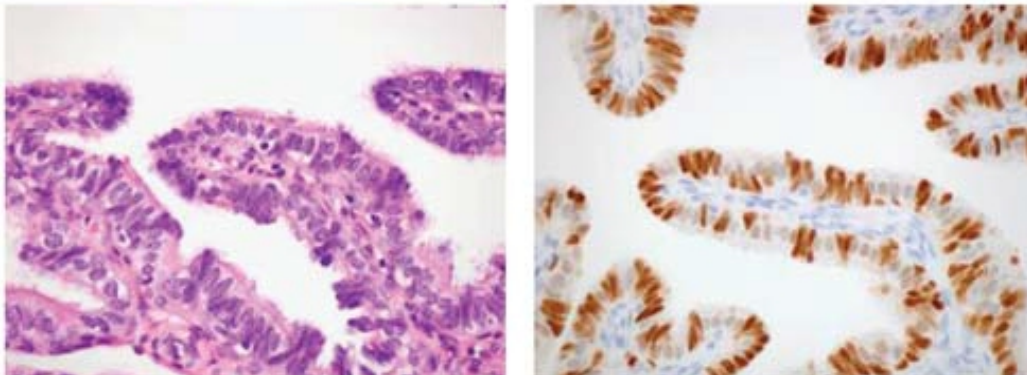

Figure 6. ***PAX8 is positive in secretory cells of Fallopian tube fimbria but not in ciliated cells*** *(modified from Laury et al. 2011)*
(left) Haematoxilin and eosin staining of the Fallopian tube fimbria; (right) Immunohistochemistry (IHC) for PAX8 in the Fallopian tube fimbria.

Consistent with its role as a lineage marker (Bowen et al. 2007, Cheung et al. 2011), PAX8 expression is retained in the Fallopian tube cells during the process of secretory cell malignant transformation, both in STIC lesions and in the vast majority of HGSCs (Laury et al. 2010, Laury et al. 2011, Tacha et al. 2011) (Figure 7).

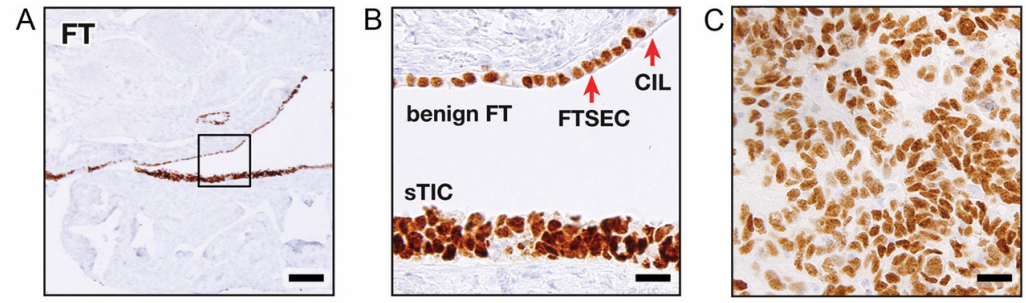

Figure 7. ***Expression of PAX8, a specific Müllerian lineage marker, during Fallopian tube malignant transformation*** *(modified from Perets et al., 2013)*
(A, B) Immunohistochemistry (IHC) for PAX8 in the human FTE is shown for the benign epithelium (top) and STIC (bottom). The square area in (A) is shown at a higher magnification in (B). PAX8 positive FTSEC and PAX8-negative ciliated cells (CIL) are demarcated by red arrows. (C) IHC for PAX8 in human HGSC.

PAX8, initially identified in normal cells originating in Müllerian ducts, is also present in ovarian neoplasia (Tong et al. 2011, Tacha et al.2011), and it is characteristic for the epithelial phenotypes (serous, clear cell, and endometrioid) but mesothelial cells stain negative for PAX8 (Figure 8).

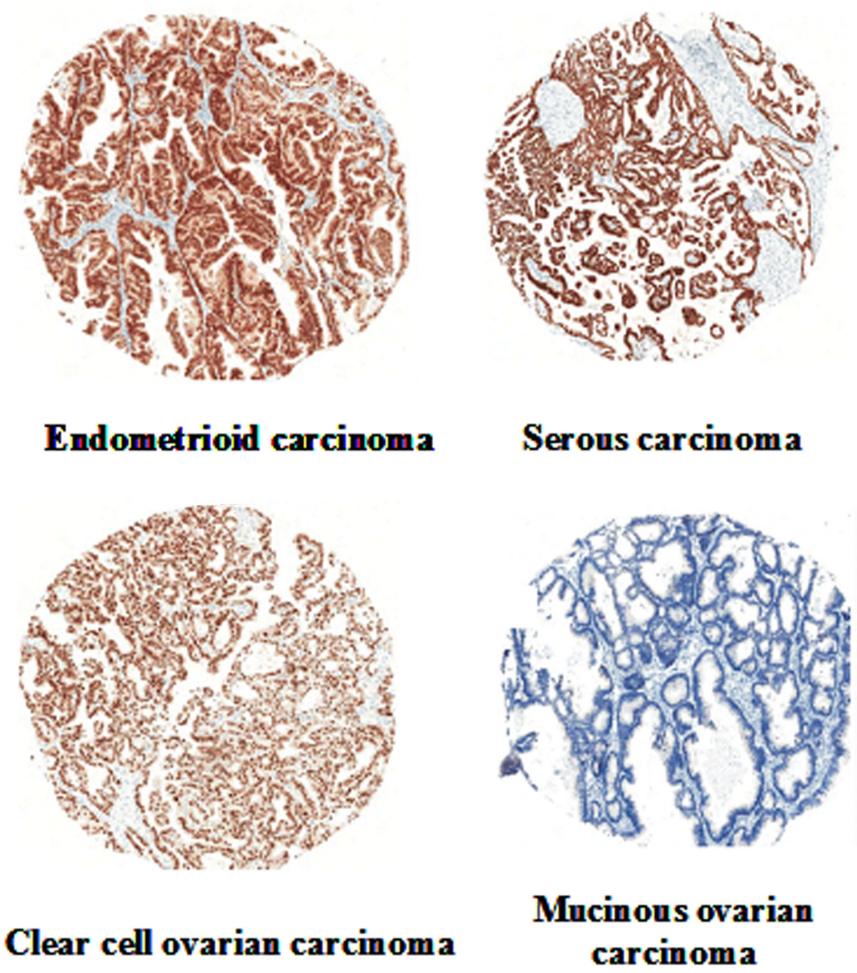

Figure 8. ***Immunohistochemistry staining of PAX8 in EOC*** *(modified from Liliac et al., 2013)*

Consequently, the positive expression of PAX8 strongly supports the origin of ovarian carcinoma in the fimbrial area of Fallopian tubes or in endometriosis foci (Mhawech-Fauceglia et al 2012). Moreover, PAX8 expression allows the differentiation between Müllerian and non-Müllerian origin in the case of ovarian metastatic carcinomas that could derive from a primary tumor in pancreas, colon or mammary gland. The ovary is a common site for the formation of metastases and the breast is one of the most common sources. Ovarian and breast cancers develop from hormonally responsive tissues, comprise various histopathological subtypes, and exhibit considerable variability in clinical manifestations and prognosis. Metastatic breast

carcinoma is known to morphologically mimic primary ovarian carcinoma, resulting in difficulty in distinguishing between these forms of cancer. PAX8 is stained in none of the breast and almost all ovarian cancer samples, indicating that PAX8 is a valuable marker for the differential diagnosis of ovarian and breast cancer (Nikiforov 2011). Ovarian cancers are frequently associated with metastases, which are commonly found in peritoneal fluids (Edge et al 2010). Thus, PAX8 positive staining appears to be highly specific and sensitive for detecting metastatic ovarian serous carcinoma in cytological preparations, and may prove useful for distinguishing these cells from mesothelial cells in fluid cytology (McKnigh et al 2010). In addition, PAX8 detection is useful for recognition of metastatic carcinomas in pelvic washings, particularly in cases with suspicious cytology (Xiang et al 2012).

There are gaps in the literature with respect to direct links between PAX gene expression and mechanisms of tumor generation. The available data indicate that PAX genes in adult tissues may not be themself oncogenes but they may contribute to the malignant phenotype by sustaining abnormal cell proliferation. In agreement with previous data that indicated the role of PAX genes in cell survival (Bouchard et al. 2000, Ostrom et al 2000, Porteous et al 2000), the analysis of kidneys obtained from PAX2-PAX8 double knochout mice shows an increase in apoptotic cell death (Narlis et al 2007). Similarly, the silencing of PAX8 in differentiated thyroid cells highlighted its new role in the regulation of cell survival and proliferation (Di Palma et al. 2013).

In conclusion, numerous evidences demonstrate the importance of specific PAX proteins such as PAX8 as prognostic markers for cancers and/or potentially targets for novel anti-cancer therapies.

2. AIMS OF THE STUDY

Recent studies have shown that Fallopian tube secretory cells expressing PAX8 are the origin of the high-grade serious carcinoma(HGSC),a subtype of ovarian cancer, that account for 90% of the cases among these tumors.

A genome-wide screening of pooled shRNAs in 25 ovarian cancer cell lines identified the transcription factor PAX8 as amplified in primary high-grade ovarian tumors and essential for survival and proliferation of this type of tumor (Cheung et al., 2011). Furthermore, recent studies from our research group have demonstrated that PAX8 is involved in cell survival and proliferation of thyroid differentiated epithelial cells (FRTL-5) (Di Palma et al., 2013). Thus the main objective of this study was to investigate the role of PAX8 in the tumorigenic phenotype of ovarian cancer cells.

To this aim we investigate whether interfering with PAX8 expression in cellular models of ovarian cancer would translate into a reduction of cell survival, migration and *in vivo* tumorigenicity. In particular, the specific aims of this research included: *in vitro* evaluation of PAX8 involvement in cellular proliferation, migration and invasion of SKOV-3 ovarian cancer cell line stably silenced for PAX8; *in vivo* assessment of the tumorigenic properties of PAX8 through injection of PAX8 silenced clones into nude mice and evaluation of the tumor growth. Finally, the last aim was the identification of potential PAX8 target genes in the ovarian cancer and Fallopian tubes cells by trascriptome analysis of transiently PAX8 silenced SKOV-3 and FT-194 cells.

Overall, the findings of this study may contribute to a better understanding of the role of PAX8 in the progression of ovarian epithelial carcinomas and to the identification of new direct or indirect targets of PAX8, thus opening the way to novel diagnostic and therapeutic approaches for such aggressive cancers.

3. MATERIALS AND METHODS

3.1 Cell culture and transfection assay

SKOV-3, TOV-21G, OVCAR-3 and TOV-112D ovarian cancer cells were grown in RPMI medium (Euroclone) supplemented with 10% foetal bovine serum (FBS). Fallopian tube cell line FT-194 was grown in F12/DMEM medium containing 2% Ultroser G Serum Substitute for Animal Cell Culture (USG).

For stable transfection experiments, FUGENE 6 reagent (Promega) was used for SKOV-3, according to the manufacturer's instructions. The DNA/FUGENE ratio was 1:3 in all the experiments. Forty-eight hours later, transfected SKOV-3 cells were selected in the presence of 0.4 μg/ml of puromycin (Sigma-Aldrich). For stable transfection experiments, cells were plated at 5×10^5 cells/100-mm tissue culture dish 24 h prior to transfection with 2 μg of proper DNA.

For transient transfections, Lipofectamine (Invitrogen) was used for FT-194 and SKOV-3 cell line according to the manufacturer's instructions. Briefly, cells were plated at a density of $2x10^4$ per well and were transfected in triplicate with 5 nM of PAX8 siRNA or siRNA non targeting.

3.2 shRNA and siRNA

Five shRNA targeting PAX8, Mission shRNA lentiviral plasmids (SHCLNG-NM_003466, Sigma-Aldrich) and MISSION non-targeting shRNA control vector (SHC002, Sigma-Aldrich) were used for the stable transfection of SKOV-3 cells.

PAX8 siRNA (silencer select Pre-designed siRNA ID:s15403, and silencer select negative control #1 siRNA, AMBION) were used for transient transfection of FT-194 cells and SKOV-3 cells.

3.3 RNA extraction, RT-PCR analysis and quantitative real time PCR

Total RNA was prepared using TRIzol reagent (Invitrogen) according to the manufacturer's directions. Total RNA (1μg) was retrotranscribed using iScript cDNA Synthesis kit (Bio-Rad Laboratories). The cDNAs were used for both classic PCR reaction and real-time PCR. The real time PCR analysis was performed using iCycler-iQ real-time detection system and SYBER green chemistry (Bio-Rad Laboratories). To design the primers for qRT-PCR assays, the Primer Express software was used. The primers sequences used are the following:

HUMAN	Oligonucleotide sequence
ABL Fw	5'tggagataacactctaagcataactaaagg3'
ABL Rw	5'ctgctttatggcgaagggtg3'
PAX8 Fw	5'cccttccaacacgccact3'
PAX8 Rw	5'ctgctttatggcgaagggtg3'

Reactions were carried out in duplicate in three independent experiments. For each gene, the data reported are the mean value ±SD of three independent experiments, normalized by the expression of the housekeeping gene, and expressed as a percentage of the value measured in parental FT-194. To calculate the relative expression levels, we used the $2^{-DD_{CT}}$ method (Livak and Schmittgen 2001).

3.4 Protein extract and immunoblotting

Cells were washed twice with ice-cold PBS and lysed in a buffer containing 10 mM HEPES (pH 7.9), 400 mM NaCl, 0,1 mM EGTA (pH 7.8), 5% glycerol, 1 mM dithiothreitol, and 1mM phenylmetilsulfonil fluoride. The protein concentration was determined using the Bio-Rad protein assay (BioRad Laboratories).

For Western blotting analysis, proteins were separated on SDS-PAGE, and blotted onto PVDF membranes (Immobilon-P, Millipore). The primary antibodies used were: rabbit polyclonal anti-PAX8, actin (Santa Cruz Biotechnology) mouse monoclonal anti-GAPDH (Santa Cruz Biotechnology). Secondary antibodies anti-rabbit or anti-mouse IgG-HRP whole antibody (GE-Healthcare) were used as suggested by manufacturers. The filters were developed using an enhanced chemiluminescence (ECL) detection method (Pierce) according to the manufacturer's instructions.

3.5 Immunofluorescence and confocal laser scanning microscopy

Cells were grown directly on glass coverslips for 48-72 h, fixed in 4% paraformaldehyde solution in PBS for 20 min at room temperature, treated for 20 min with 50 mM solution of NH_4Cl in PBS, permeabilized for 10 min in 0.2% Triton X-100 in PBS, and incubated for 60 min in 0.5% BSA (bovine serum albumin) in PBS. The coverslips were subsequently incubated at 4°C for 1 h with rabbit polyclonal anti-PAX8 diluted 1:1000 in 0.5% BSA in PBS and, after PBS washing, incubated for 30 min with Alexa Fluor-594 goat anti-rabbit IgG-TRITC (Vinci Biochem) diluted 1:200 in 0.5% BSA in PBS. After final washings with PBS, the coverslips were mounted on a microscope slide using a 50% solution of glycerol in PBS. Images were collected with a Zeiss LSM 510 confocal laser scanning microscope, equipped with a 543 nm HeNe laser, and a Plan-Apochromat 63/1.4 oil immersion objective. Emitted fluorescence was detected using LP 560 long pass filter for TRITC.

3.6 Cell proliferation assay

SKOV-3, SKOVCtrl-, siCl32, and siCl48 cells were plated at 8×10^4 cells in 60-mm plate and every day cells were counted for 6 days using a Bio-rad instrument for cell count (TC-10).

3.7 Wound-healing assay

Confluent SKOVCtrl-, siCl32, and siCl48 cells plated on tissue culture dishes were grown until confluent and wounded by manual scratching with

200-µl pipette tip, washed with PBS and incubated at 37°C in complete media. At the indicated time points, phase contrast images at specific wound sites were captured.

3.8 *In vitro* invasion assay

Cell invasion assay was examined using a reconstituted extracellular matrix (Matrigel; BD Biosciences). Filters (8 µm pore size) on the bottoms of the upper compartment of the transwells (6,5 mm; Corning) were coated with 2 mg/ml of Matrigel. 2×10^5 cells were suspended in 100 µl of RPMI with 0.2% FBS. The cells were then plated onto the coated wells and incubated at 37°C for 16 h. Medium in the lower compartment was supplemented with 5% FBS as a chemoattractant. Non-invading cells were removed from the top of the wells with a moistened cotton swab. Cells that penetrated the membrane were fixed with 11% glutaraldehyde and stained with 0.1% crystal violet. The concentration of solubilized crystal violet in 10% acetic acid was evaluated as absorbance at 590 nm.

3.9 Anchorage-independent growth in soft agar

Cells were mixed in RPMI 2X (Sigma-Aldrich Aldrich), tryptose phosphate buffer, and 1.25% of Noble Agar (Difco Laboratories Inc.), and plated in 60-mm dishes on the top of 1% agar base. The colonies were allowed to grow in incubator at 37°C, 5% CO2 for 2 to 3 weeks. The images of cell colonies were captured with an inverted microscope.

3.10 Animal experiments

All animal studies were conducted at Biogem Scarl Ariano Irpino, AV (Italy), Preclinical Research and Development Service. Nude female NOD-SCID mice (NOD-CB17/PRKDC/J) were purchased from Charles River Laboratories International, Wilmington, MA. To generate xenografts, human ovarian cancer cells were cultured in RPMI with 10% heat-inactivated FBS. 24 six-week-old nude female NOD-SCID mice were randomly assigned to four groups: the SKOV-3 group (n = 6), SKOV-3Ctrl- group (n = 6), siCl32 group (n = 6) and siCl48 group (n = 6). They were injected subcutaneously in the both flanks with 7×10^6 cells suspended in 0.2 ml PBS/Matrigel Matrix GF (1:1) (BD Biosciences). Mice were daily monitored for clinical signs and mortality. Body weight recordings were carried out weekly. Tumor growth was measured twice a week with a Mitutoyo caliper. The formula TV (mm^3) = [length (mm) × width $(mm)^2$]/2 was used. At the end of the study mice were sacrificed by cervical dislocation and tumors were collected.

3.11 RNA sequencing

Three independent silencing experiments were performed for each condition (si-PAX8 or si-CTR) and were used for RNA sequencing analysis (RNA-seq). Three µg of total RNA extracted from the three biological replicates using the RNeasy Mini kit (Qiagen), from FT-194 and SKOV-3

cells, 24 h after transfection, were sent to the Service Analysis Genomix4Life of Salerno University. RNA samples were sequenced using the Illumina TruSeqTM system (Illumina) according to manufacturer's protocols.

3.12 Data analysis

To perform differential expression analysis, FASTQ files were processed for sequence quality check using FASTQC. The TopHat2 software was used to align the raw RNA-seq FASTQ short reads to the human reference genome (GRCh37/hg19). Counting of the reads for each gene was performed using HTSeq (version 0.6.0). The Bioconductor package edgeR was used for differential gene expression analysis and genes exhibiting a $\log_2$ fold change $\geq$ 0,6 and p-value (FDR) $\leq$ 0.05 were considered differentially expressed in FT-194 and SKOV-3 cells. Gene ontology (GO) and pathway functional class scoring were performed using the Gene Set Analysis Toolkit V2 (Zhang et al, 2004).

The promoter regions of genes co-regulated after PAX8 silencing were analyzed in order to recognize DNA binding motifs for both PAX8_01 and PAX8_B matrices. Genes mostly expressed in FT-194 and SKOV-3 cells were selected for the transcription factors binding site (TFBS) analysis using the web-based PASTAA (predicting associated transcription factors from annotated affinities) method (Roider et al 2009), which utilizes the prediction of binding affinities of a transcription factor to promoters. The list of downregulated and upregulated genes were ranked respectively according to the prediction of binding affinity of their promoter regions to the PAX8 binding sites, with the following criteria: range for promoter region from -2000 to 0 at either side of the transcription start site, conserved mouse/human sequence block, maximum affinity across promoter range.

4. RESULTS

4.1 PAX8 expression in human ovarian cancer cell lines

The expression of PAX8 in human ovarian cancer cell lines was examined by RT-PCR and Western Blotting analysis. Total RNA was extracted from SKOV-3 (serous carcinoma), TOV-21G (clear cell carcinoma), TOV-112D (endometrioid carcinoma) and OVCAR-3 (serous carcinoma) cells and 1μg of RNA was used to synthesize the respectively cDNAs that were used for RT-PCR with specific primers for PAX8. As shown in Figure 9A, PAX8 is expressed in all cell lines with the exception of TOV-112D. In addition, we evaluated the expression of PAX8 in ovarian cancer cell line by Western blot analysis of protein extracts prepared from SKOV-3, TOV-21G, TOV-112D and OVCAR-3 ovarian cancer cells. As shown in Figure 9B, the polyclonal antibody that specifically recognizes PAX8 revealed the presence of a protein of the predicted relative molecular mass in SKOV-3, TOV-21G and OVCAR-3 ovarian cancer cell lines, whereas it resulted to be undetectable in TOV-112D cells.

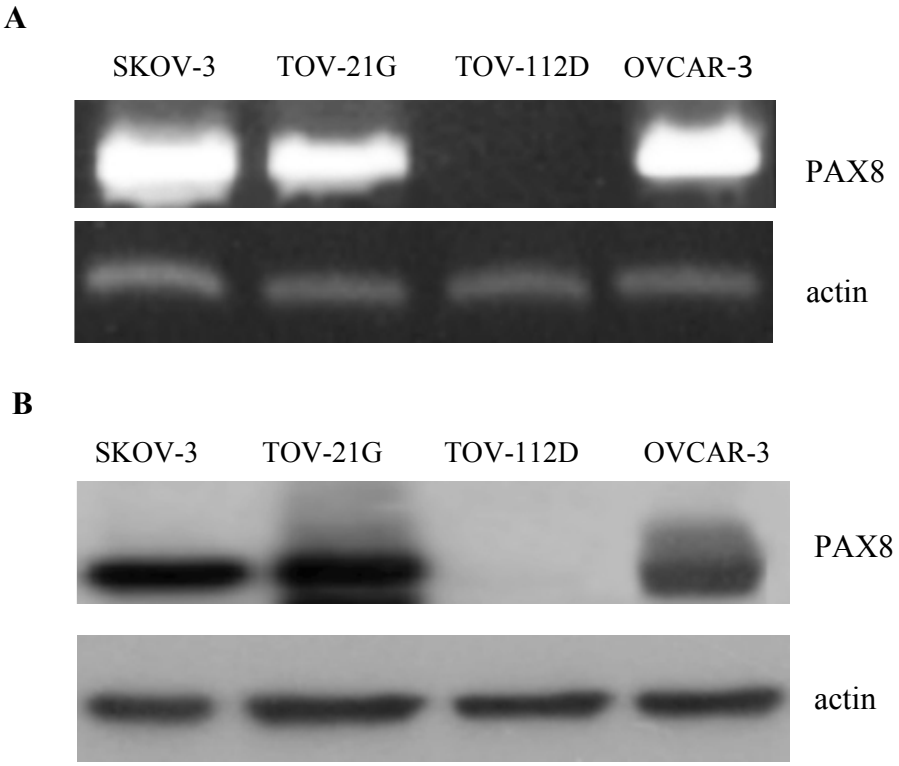

Figure 9. ***Expression of PAX8 in human ovarian cancer cell lines***
A. RT-PCR was performed using total RNA from SKOV-3, TOV-21G, TOV-112D and OVCAR-3 ovarian cancer cells and β-actin mRNA was amplified as control
B. Protein extracts from SKOV-3, TOV21G, TOV112D and OVCAR-3 cells were subjected to SDS-PAGE and Western blot with a specific anti-PAX8 antibody. The hybridization with actin assessed the protein uniform loading and integrity.

Furthermore, the subcellular localization of PAX8 protein in these cell lines was analyzed by immunofluorescence. To address this issue, SKOV-3, TOV-21G and TOV-112D cells were grown directly on glass coverslips and were processed for immunofluorescence analysis with a specific anti-PAX8 polyclonal antibody. The signals from the PAX8 immunostaining were acquired at high resolution by line-wise scanning microscopy. PAX8 was observed exclusively in the nuclei of SKOV-3 and TOV-21G cells as expected being PAX8 a transcriptional factor, while TOV-112D resulted negative at the staining for PAX8 as expected on the basis of the results obtained from RT-PCR and Western blot analysis (Figure 10).

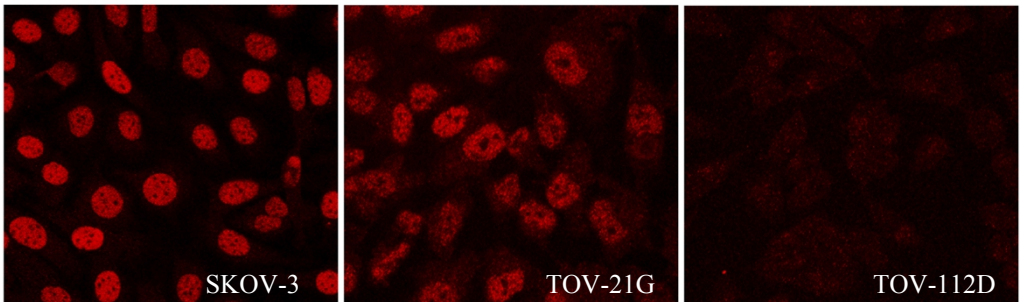

Figure 10. ***PAX8 immunofluorescence staining in ovarian cancer cells lines***
SKOV-3, TOV-21G and TOV-112D cells were grown directly on glass coverslips and stained for immunofluorescence with the anti-PAX8 antibody. The fluorescence signals were acquired at a confocal microscope by line-wise scanning. The staining for PAX8 was detected only in the nuclei of the cells.

4.2 Silencing of PAX8 expression in SKOV-3 cells

To investigate the role of PAX8 in the tumorigenic properties of ovarian cancer cells, we silenced its expression in the SKOV-3 cell line using shRNA plasmid vectors. In particular, SKOV-3 cells were transfected with 2 µg of a pool of five plasmid vectors, each containing an shRNA against different region of PAX8 cDNA, and with the empty vector as control (see Materials and Methods). Forty-eight hours after transfection, the selection with the specific antibiotic, puromycin, was started, and about sixty independent clones were isolated. The silencing of PAX8 expression in the stable clones was examined by Western blotting analysis of protein extracts prepared from each clone. The screening analysis of all the clones allowed the identification of two clones, named siCl32 and siCl48, with different levels of PAX8 expression silencing, as shown in Figure 11A. These results were confirmed by immunofluorescence followed by confocal fluorescence microscopy. As shown in Figure 11B, the signal from the PAX8 protein acquired at high resolution by line-wise scanning indicated that the siCl32 and siCl48 clones expressed different silencing levels of PAX8 with respect to the SKOV-3 control (SKOVCtrl-). These clones were selected for all further studies.

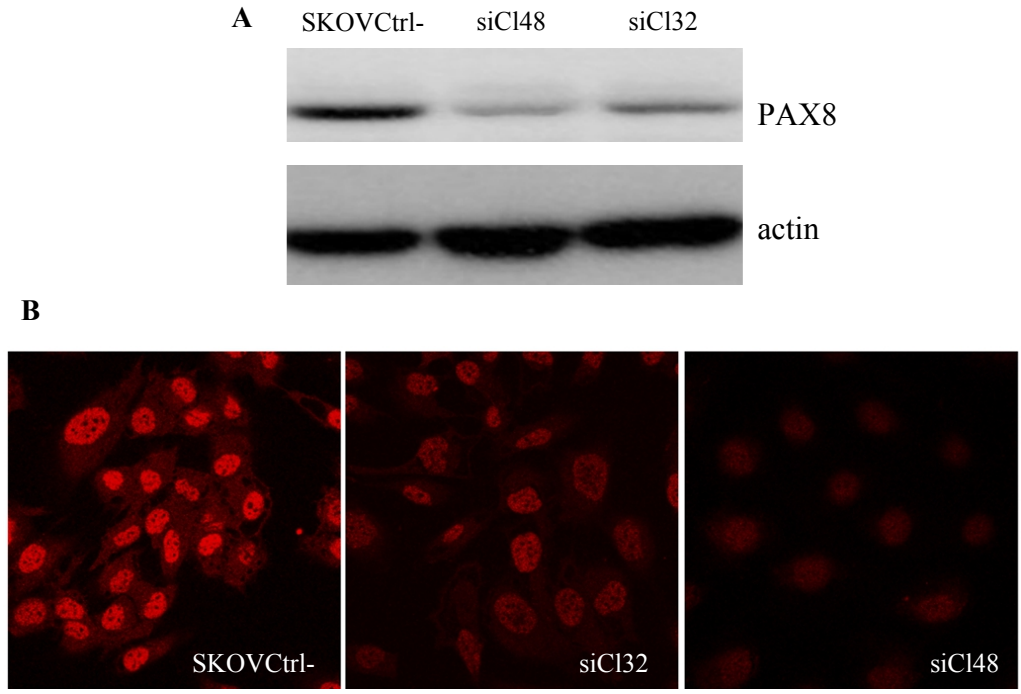

Figure 11. ***Western blotting and immunofluorescence for PAX8 in two independent representative clones (siCl32 and siCl48)***

A. Total protein extracts prepared from each stable clone were separated on SDS-PAGE and subjected to Western blotting analysis with a specific PAX8 antibody. The hybridization with actin assessed the protein uniform loading and integrity. B. Stable SKOVCtrl-, siCl32 and siCl48 clones were processed for the immunofluorescence assay with a specific PAX8 polyclonal antibody.

4.3 Effects of PAX8 silencing on proliferation, migration and invasion of SKOV-3 cells

To examine whether PAX8 expression could directly contribute to the tumorigenicity of ovarian cancer cells, we analyzed whether PAX8 silencing was able to modify the oncogenic properties of SKOV-3 cells. First, we analyzed the proliferation rate of the above mentioned SKOV-3 stable clones by growth curves experiments. 80,000 cells of each stable clones were plated at time (t=0) and counted each day for six days. As shown in Figure 12, PAX8 expression confers a proliferative advantage to SKOV-3 cells; in fact, the proliferation rate of each clone resulted PAX8 dose-dependent and was reduced compared to SKOVCtrl-, and SKOV-3 wild-type cells.

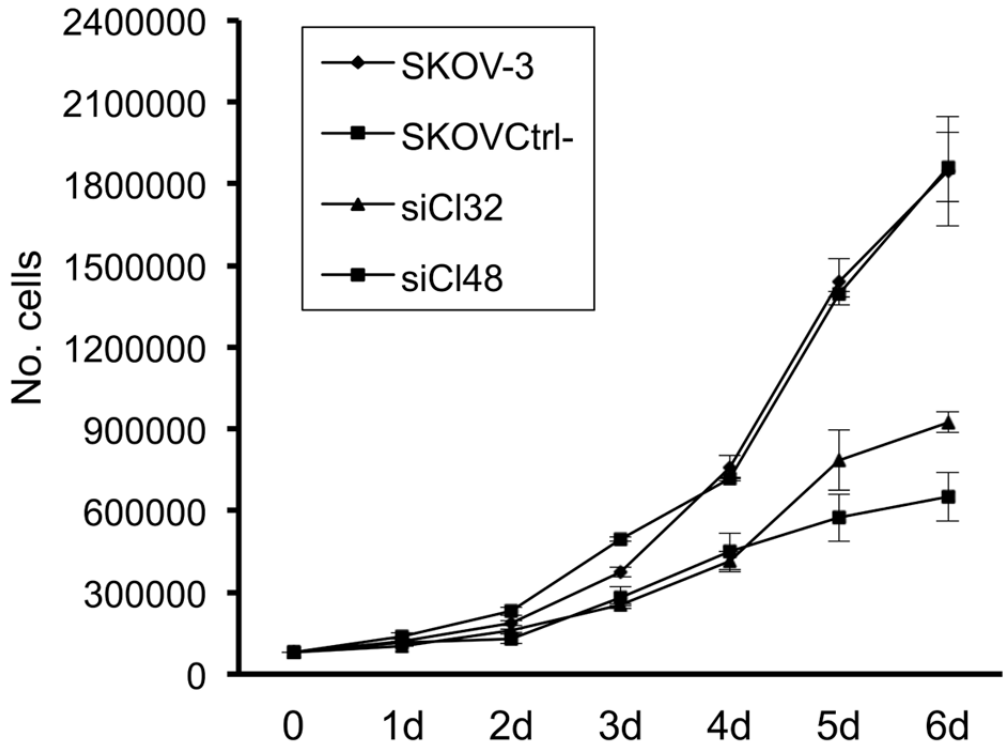

Figure 12. ***PAX8 silencing in SKOV-3 cells inhibits cell proliferation***
Growth curves of SKOV-3, SKOVCtrl-, siCl32, and siCl48 cells are shown. Triplicate of 8 ×104 cells were seeded into 60-mm plate. Cell numbers were counted on days 1, 2, 3, 4, 5 and 6 after seeding.

Moreover, in order to investigate the role of PAX8 in cell migration and invasion, wound healing and transwell assays were performed. In the wound healing assays, we compared the cell motility of the siCl32 and siCl48, independent stable clones, with that of SKOVCtrl- cells transfected with the empty vector. After 8 h, the area of the wound was significantly recovered by migrating SKOVCtrl- cells and after 24 h the wound area was completely recovered, as shown in Figure 13. In contrast, the motility of the siCl32 and siCl48 clones stable silenced for PAX8 at 8 and 24 h was significantly decreased, as shown in Figure 13. These results well correlate with PAX8 expression levels, suggesting that PAX8 silencing significantly reduces the migration of SKOV-3 cells.

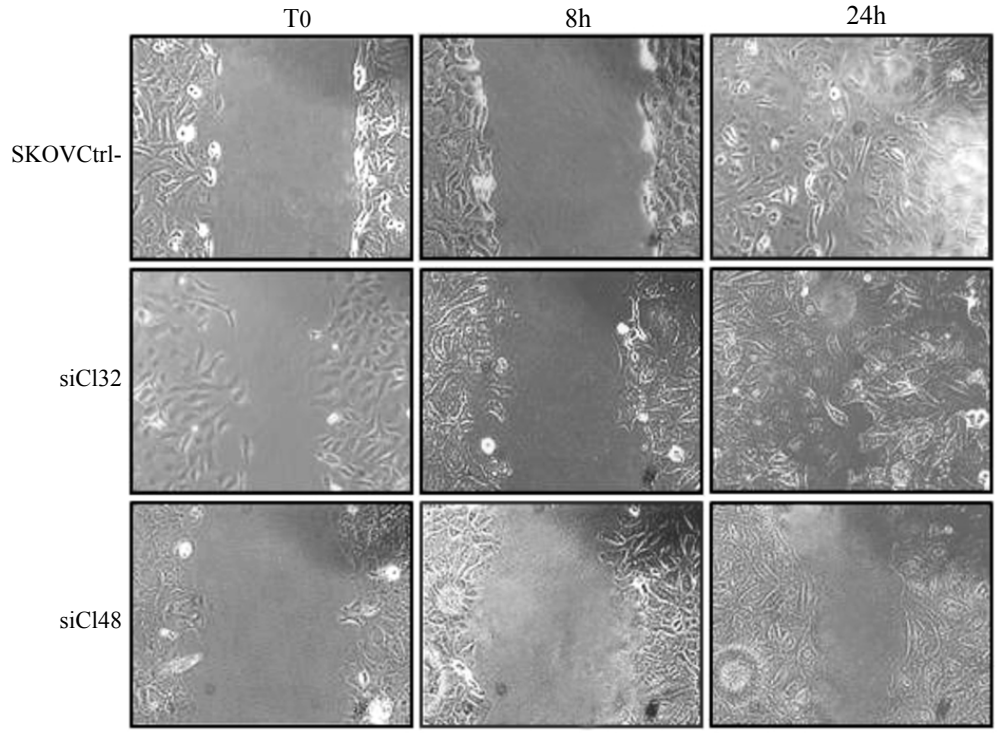

Figure 13. ***PAX8 silencing in SKOV-3 cells reduces cell migration***
Wound-healing migration assay for SKOVCtrl-, siCl32, and siCl48 was performed. The healing of wounds by migrated cells at time 0, 8 and 24 h is shown. The siCl32 and siCl48 cells migrate slower than SKOVCtrl-.

Finally, to further study the effect of PAX8 silencing in SKOV-3 cells in *in vitro* invasion assay, we performed a transwell migration assay in which cells were seeded in serum-free medium on the top chamber of a 2-chamber transwell cell culture plate. Colorimetric evaluation of the cells migrated to the lower chamber revealed that the invasiveness of the siCl32 and siCl48 was decreased with respect to SKOVCtrl- clone, as represented in Figure 14. These results indicate that PAX8 is involved in cell migration and invasion capabilities of ovarian cancer cells.

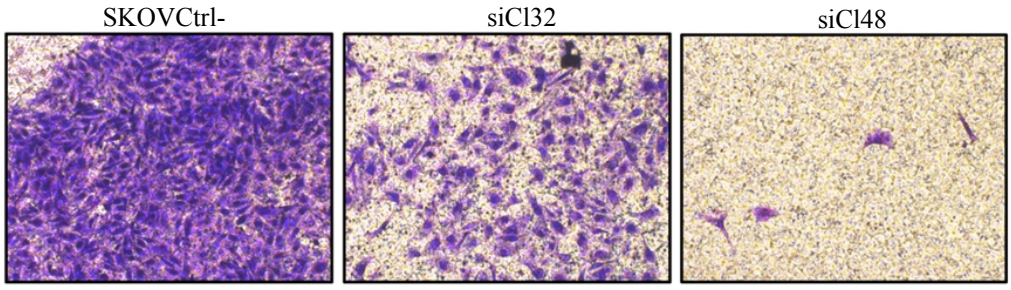

Figure 14. ***PAX8 silencing in SKOV-3 cells reduces in vitro invasion***
Matrigel invasion assay of SKOVCtrl-, siCl32, siCl48 clones after 16 h.

4.4 Effects of PAX8 silencing on tumorigenesis *in vivo* and *in vitro* of SKOV-3 cells

To investigate the role of PAX8 in the tumorigenicity of SKOV-3 cells *in vitro* and *in vivo* we performed soft agar and nude mice assays. We seeded the cells in plates containing 1.25% noble agar. After incubation at 37°C for 21 days, we observed that the siCl32 and siCl48 clones were not able to grow efficiently in soft agar and to form colonies. They formed only small aggregations of cell debris, while SKOVCtrl- clone efficiently grew on soft agar (Figure 15).

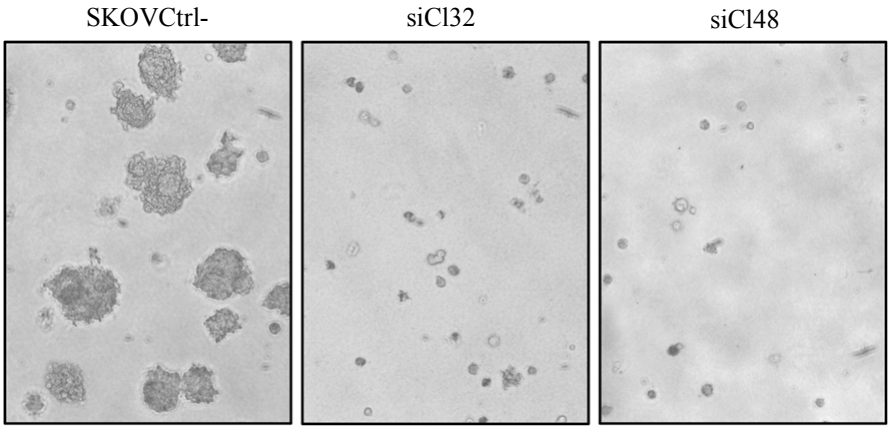

Figure 15. ***PAX8 silencing in SKOV-3 cells reduces in vitro anchorage-independent growth***
Soft agar growth assay of SKOVCtrl-, siCl32 and siCl48 clones was performed and photographed after 21 days.

These results suggested that PAX8 is essential for anchorage-independent growth of SKOV-3 cells.

Subsequently, to examine PAX8 role in *in vivo* tumorigenesis, SKOVCtrl-, siCl32, siCl48 clones and parental cells were separately injected subcutaneously into the flanks of nude mice, and the growth of the tumors was monitored. To this aim, we have grown $7x10^6$ cells of each clone, suspended in 0.2 ml PBS/Matrigel Matrix GF (1:1) and send them to the Biogem facility for injection into nude female NOD-SCID mice. Every week after implantation, the tumor size was measured with calliper and the tumor growth represented by volume was reported in Figure 16A. As shown in this figure, SKOV-3 parental cells formed tumors with the same efficiency as the SKOVCtrl- cells, while siCl32 and siCl48 cells were able to form smaller tumors with respect to SKOVCtrl- cells, as also indicated by the tumor sizes excised from the sacrificed mice (Figure 16B).

All together these data emphasize the involvement of PAX8 in *in vitro* and *in vivo* tumorigenesis.

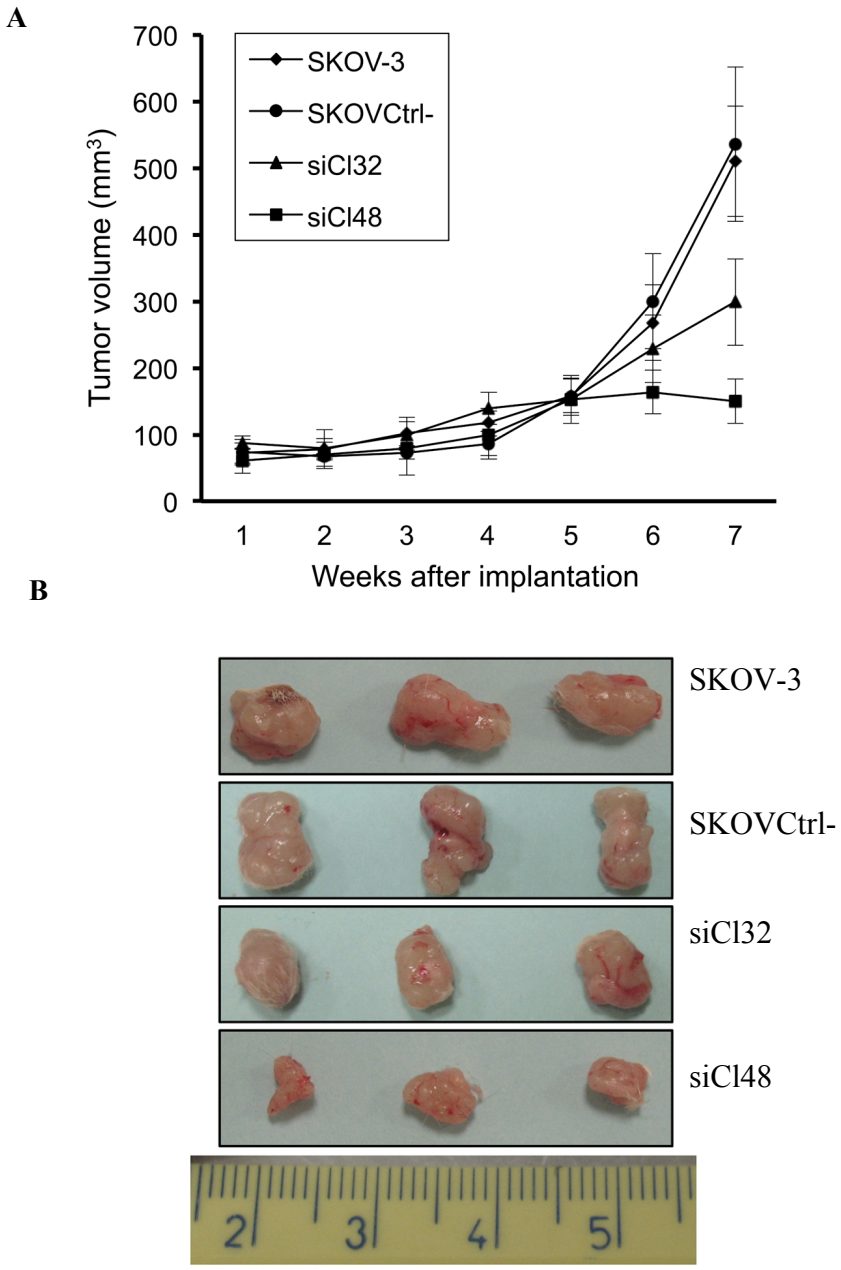

Figure 16. ***PAX8 silencing reduces tumorigenesis in nude mice***
A. Growth curves of tumor size measured for 7 weeks after injection by calliper. B representative images of the tumors from nude mice injected with SKOV-3, SKOVCtrl-, siCl32 and siCl48 cells.

4.5 PAX8 expression in human Fallopian tube cell line (FT-194)

Recent studies have demonstrated that many serous ovarian carcinomas can originate from Fallopian tubal secretory epithelial cells (Perets et al, 2013). For better understanding the potential role of PAX8 in Fallopian tube cells, we

first examined the expression of PAX8 in the FT-194 cells by Western blotting analysis. As shown in Figure 17A, the polyclonal antibody that specifically recognizes PAX8 revealed the presence of a protein of the predicted relative molecular mass in FRTL-5 cells, used as positive control, SKOV-3 and FT-194 cell lines. In addition, we evaluated the expression of PAX8 by qRT-PCR. To this end, total RNA was extracted from SKOV-3 cells and FT-194 cells, from Fallopian tube tissue (OriGene Technologies), and from normal ovary tissue (OriGene Technologies) used as positive and negative control, respectively. Figure 17B shows that PAX8 is expressed in SKOV-3 cells, FT-194 cells and Fallopian tube tissue while it is absent in the normal ovary tissue.

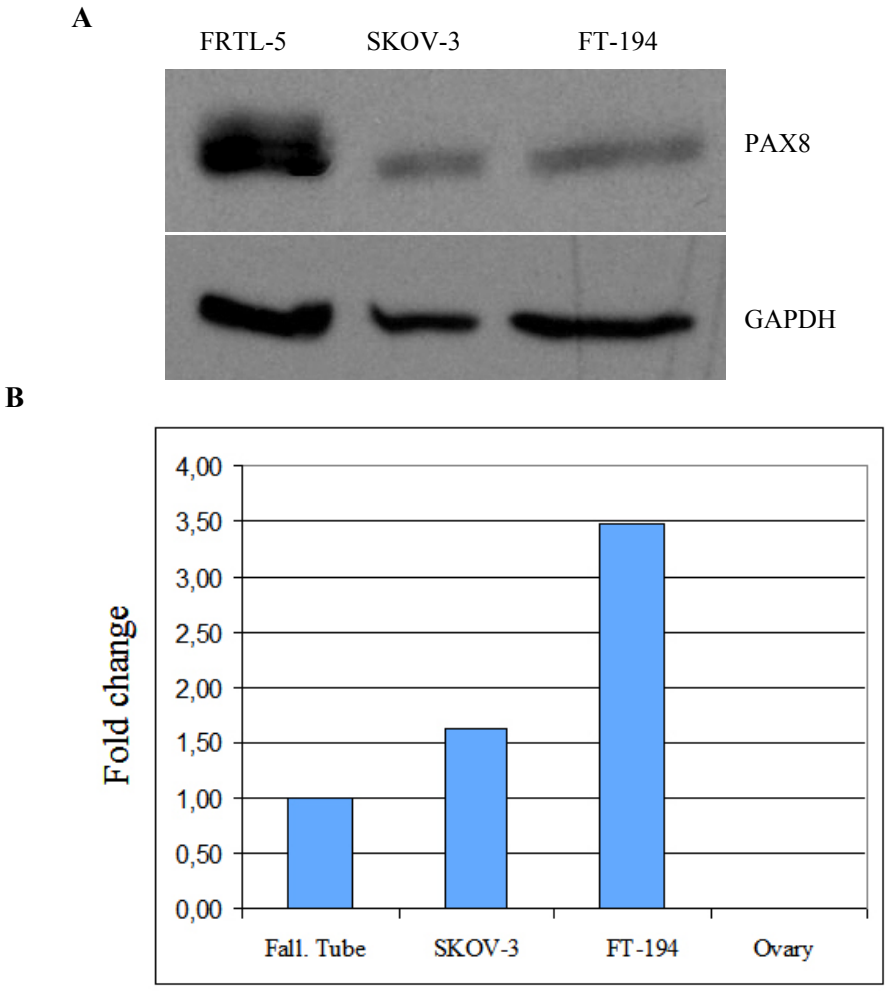

Figure 17. ***Expression of PAX8 in FT-194 epithelial fallopian tube cells***
A. Total protein extracts prepared from all cell lines were separated on SDS-PAGE and subjected to Western blot analysis with a specific anti-PAX8 antibody. The hybridization with GAPDH assessed the protein uniform loading and integrity. B. qRT-PCR analysis was performed on total RNA prepared from all the samples and the expression of PAX8 was measured.

Furthermore, the subcellular localization of PAX8 protein in FT-194 cell line was analyzed by immunofluorescence. To address this issue, SKOV-3 cells used as a positive control, and FT-194 cells were grown directly on glass coverslips and all cells were processed for immunofluorescence assay with a polyclonal anti-PAX8 antibody. The signals from the PAX8 immunostaining were acquired at high resolution by line-wise scanning microscopy. The results indicated that PAX8 was present exclusively in the nuclei of SKOV-3 and FT-194 cells as expected being PAX8 a transcriptional factor (Figure18).

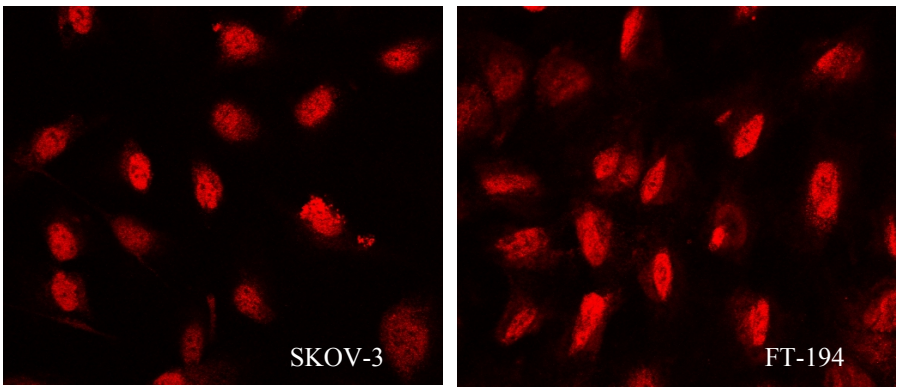

Figure 18. ***PAX8 immunofluorescence staining in FT-194 cells***
SKOV-3 and FT-194 cells were grown directly on glass coverslips and stained for immunofluorescence assay with the anti-PAX8 antibody. The fluorescence signals were acquired at a confocal microscope by line-wise scanning. The staining for PAX8 was detected only in the nuclei of the cells.

4.7 Genome-wide identification of PAX8 target genes in SKOV-3 and FT-194 cells through RNA Sequencing Analysis

To investigate the role of the transcription factor PAX8 in Fallopian tube differentiation and in ovarian cancer disease, we transiently silenced PAX8 expression using the specific PAX8 siRNA.

In particular, FT-194 and SKOV-3 cells were transfected, with 5 nM of specific PAX8 siRNA or 5 nM of scramble siRNA used as control (see Materials and Methods). The decrease of PAX8 expression was detected 24 h after transfection by qRT-PCR and Western blotting (Figure 19).

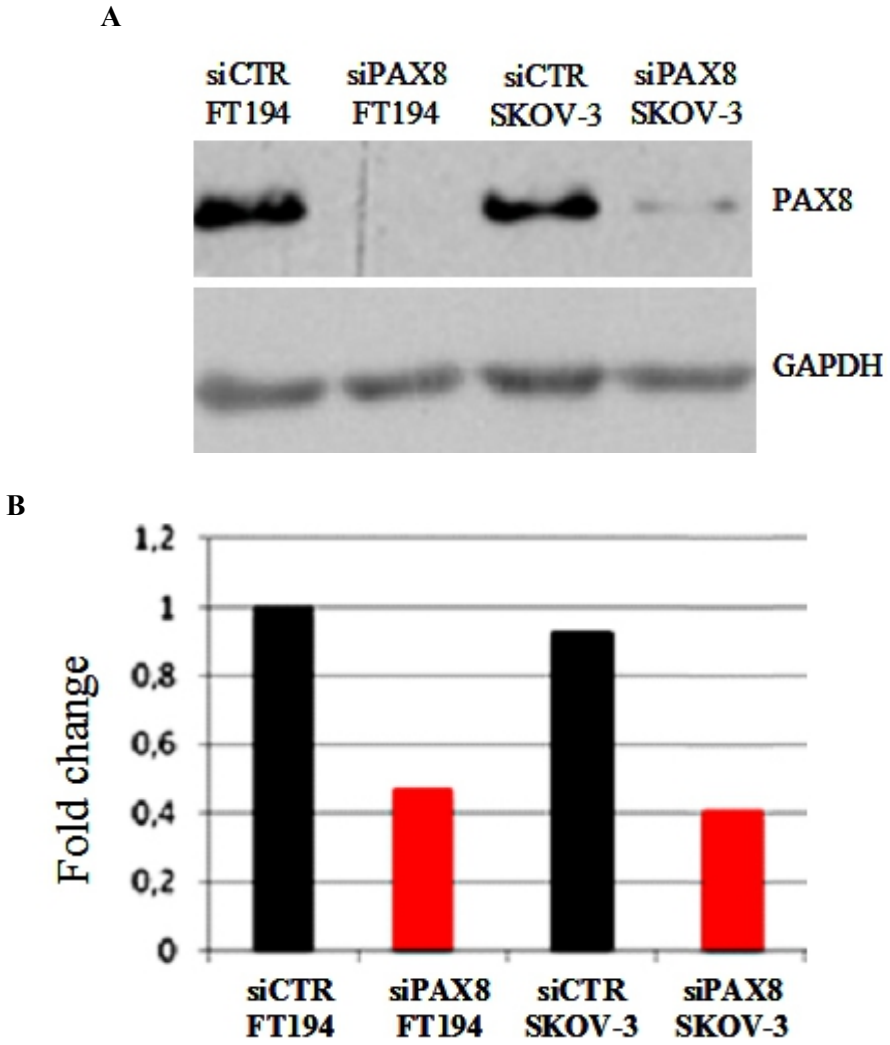

Figure 19. ***Transient silencing of PAX8 expression in FT-194 and SKOV-3 cells***
A. Total protein extracts prepared from FT-194 and SKOV-3 cells were separated on SDS-PAGE and subjected to Western blot analysis with a specific anti-PAX8 antibody. The hybridization with GAPDH assessed the protein uniform loading and integrity. B. qRT-PCR analysis was performed on total RNA prepared from all the samples and the expression of PAX8 was measured.

The RNAs obtained from SKOV-3 and FT-194 cells 24 h after transfection with siRNA against PAX8 or scrambled sequence siRNA were used to perform RNA sequencing analysis (RNA-Seq), in order to identify genes differentially expressed in PAX8 knockdown SKOV-3 and FT-194 samples. This study is the first RNA-Seq-based gene expression analysis of PAX8 target genes undertaken in Fallopian tube cells and ovarian cancer cells. The RNA obtained 24 h after transfection from the two cell lines was sent to the Service Analysis Genomix4Life of Salerno University for sequencing libraries generation. Three independent biological samples of siPAX8 FT-194

and SKOV-3 were submitted to IlluminaTruSeq sequencing platform, and the raw data were analyzed by Prof. Michele Ceccarelli (BioGeM s.c.a.r.l, Institute of Genetic Research "Gaetano Salvatore" Ariano Irpino, Italy). A total of 182 and 164 genes differentially expressed with a $\log_2$ Fold change ≥ 0,6 and corrected *p*-value (FDR) ≤ 0.05 were obtained for SKOV-3 and FT-194 cells, respectively. In SKOV-3 cells of 182 genes, 45 were up-regulated and 137 were down-regulated as shown by the Smearplot (Figure 20A), while in FT-194 cells of 164 genes, 46 were up-regulated and 118 down-regulated (Figure 20B).

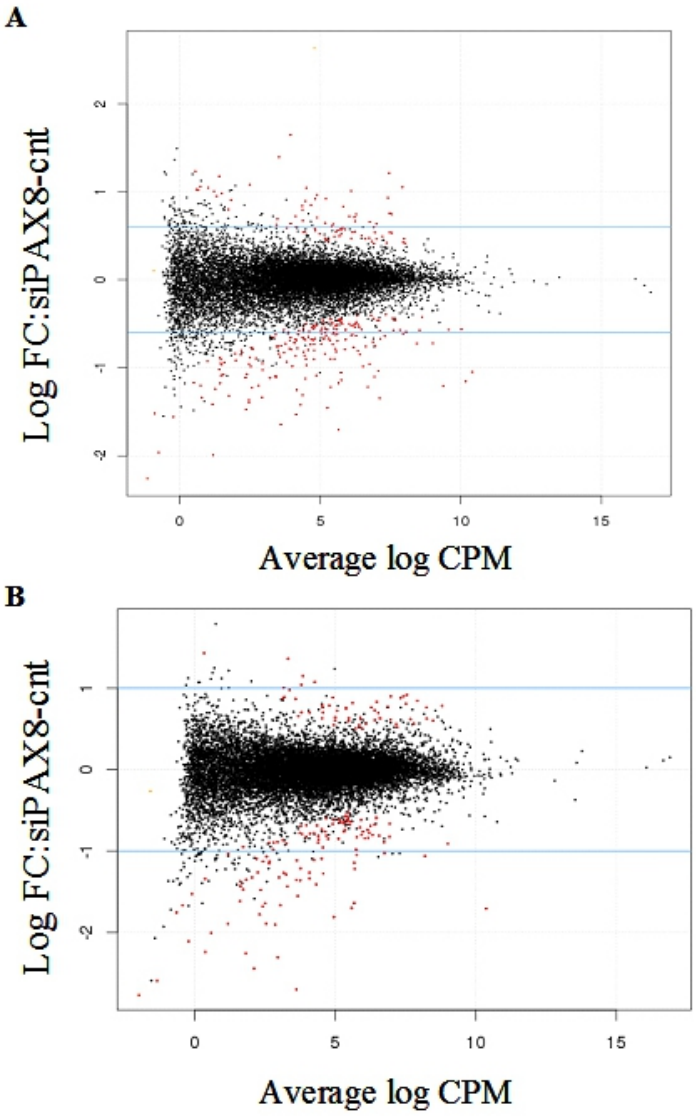

Figure 20 ***Smear plot, representing the mean of expression values among all samples (x axis) and fold change between the two conditions (y axis)***
A, SKOV-3 cells; B, FT-194 cells.

Gene Ontology (GO), used to categorize biological processes that are over-represented in PAX8 transiently knockdown SKOV-3 and FT-194 cells, demonstrated that the most affected categories were: cancer and reproductive system disease, cellular growth and proliferation, cell death and survival, and cellular movement. Accordingly, the most affected pathways included the regulation of epithelial-mesenchymal transition, p53 signalling and apoptosis, cyclins and cell cycle regulation, ovarian cancer signalling, as well as other cancer pathways.

However, it was uncertain whether these transcripts level changes were caused by direct PAX8 regulation or cascade reactions. Since the goal of our study was the identification of novel PAX8 targets in Fallopian tube cells and ovarian cancer cells, a strategy was performed to reduce the number of false positive targets by merging the data obtained from two independent experiments, in order to obtain the common genes modulated by PAX8 silencing. As shown in Figure 21, 35 up-regulated and 138 down-regulated genes were common in two cell lines.

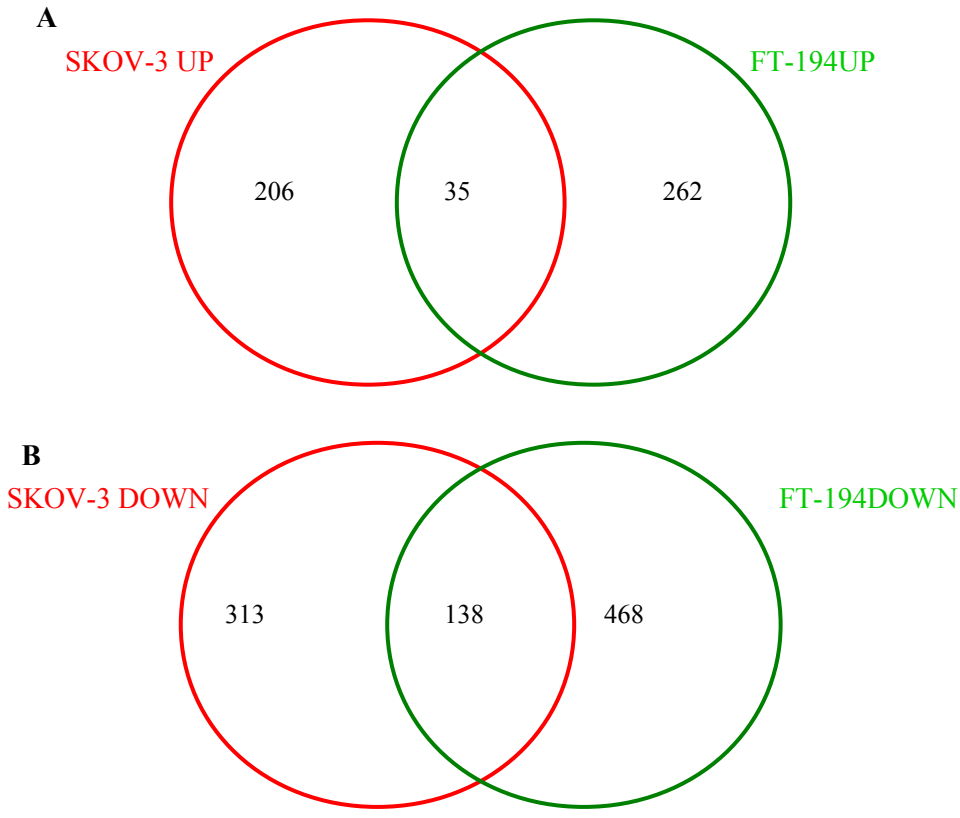

Figure 21 ***Venn diagram showing the comparison between PAX8 knockdown SKOV-3 and FT-194 cells***
A, up-regulated genes; B, down-regulated genes.

To identify PAX8 direct targets, the common genes were analyzed for the presence in their 5'-flanking regions of PAX8 binding consensus sequences and were ranked according to their affinity score to PAX8_01 and PAX8_B matrices ≥ 1.4 using the PASTAA Method (Roider et al, 2009). A total of 125 genes could be PAX8 putative direct targets considering a score between 0,75 to 1, and of these 20 genes showed a higher PAX8 binding score (0,99).

Pathway analysis from the 125 common genes found as putative targets of PAX8 showed that many pathways were perturbed upon PAX8 silencing in SKOV-3 and FT-194 (Figure 22). These pathways include: cell death and survival, cell cycle, reproductive system development and function, cellular growth and proliferation, organ development, cancer, reproductive system disease, tumor morphology, cell to cell signalling and interaction and cellular movement.

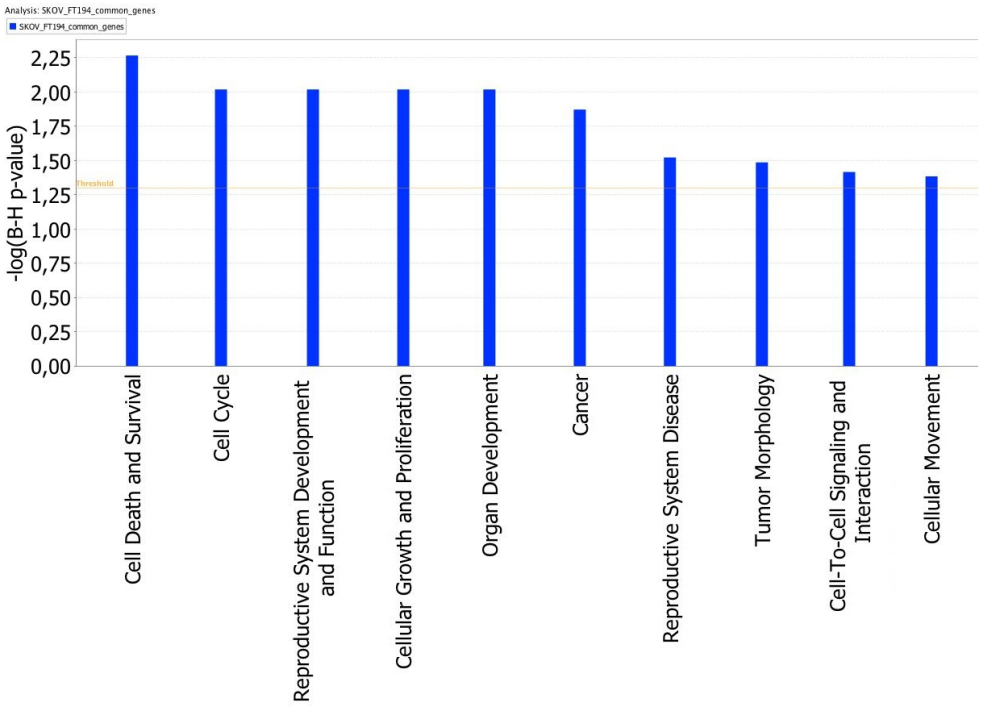

Figure 22 ***Pathway analysis of 125 common genes differentially expressed in siPAX8 SKOV-3 and FT-194.***
Pathway-Express software was used to identify the pathways most affected by the common genes.

Therefore, it is likely that in the list of the genes that resulted from the PASTAA analysis there are many interesting PAX8 targets to be further validated and analyzed to highlight several biological pathways downstream of this transcription factor.

5. DISCUSSION

Epithelial ovarian cancer is a morphologically and biologically heterogeneous disease and remains a leading cause of morbidity and mortality in female affected by cancer of the reproductive system. It accounts for approximately 3% of all cancers in women and despite considerable efforts to improve early detection and advances in chemotherapy, the highest mortality rate of ovarian cancers has markedly increased worldwide. In literature there is ample evidence that dysregulated expression or activation of specific members of the PAX family play a major role in the progression of specific cancers arising in those organ systems in which PAX proteins exert developmental functions during embryogenesis (Robson et al. 2006). However, the precise role of PAX proteins in cancer is still unclear.

Recently, a genome-scale analysis of 102 cancer cell lines identified PAX8 as a lineage-specific survival gene, highly expressed in ovarian cancer lines and amplified in a substantial fraction of primary ovarian tumors (Cheung et al. 2011). Thus, in order to elucidate the role of PAX8 in ovarian cancer, *in vitro* and *in vivo* studies were performed using different ovarian cancer cell lines and nude mice models. In particular, we first analyzed the expression of PAX8 at both mRNA and protein level in SKOV-3, TOV-21G, TOV-112D and OVCAR-3 ovarian cancer cell lines. PAX8 resulted to be expressed in all the cell lines with the exception of TOV-112D cells. We chose the SKOV-3 cell line to assess PAX8 involvement in ovarian tumorigenesis and we silenced the expression of PAX8 gene in these cells to evaluate the effect of PAX8 knockdown on the cellular proliferation, migration and invasion, that represent the main biological processes involved in cancer progression. In particular, two stable cell clones constitutively silenced by PAX8 shRNA, namely siCl32 and siCl48, were selected to study the function of the transcription factor in epithelial ovarian cancer cells. Our results demonstrated that PAX8 knockdown elicits a dramatic effect on SKOV-3 cell growth, inhibits the invasion rate of these cells through the Matrigel, and reduces the migration rate of the cells in a wound-healing assay. Interestingly, the effect of PAX8 silencing on cellular responses was more pronounced using the siCl48 clone rather than the siCl32 clone reflecting different levels of PAX8 knockdown. Similar results showing the effects of PAX8 silencing on epithelial cell survival and proliferation in the rat thyrocyte cell line FRTL-5 have been reported (Di Palma et al., 2013).

Furthermore, to study the tumorigenic effects of PAX8 silencing *in vitro* we seed both siCl32 and siCl48 clones into soft agar plates and after an incubation at 37°C for 21 days they were not able to grow efficiently in soft agar and to form colonies, thus suggesting that PAX8 is essential for anchorage-independent growth of SKOV-3 cells. To assess the ability of PAX8 to inhibit tumor growth *in vivo,* we injected siCl32, siCl48 clones and parental SKOV-3 cells into nude female NOD-SCID mice. The results obtained demonstrated a reduction of tumor growth in the mice injected with siCl32 and siCl48 clones with respect to mice injected with parental cells, thus suggesting

that PAX8 silencing is capable of reduce *in vivo* the tumor growth. The size of palpable lesions well correlated with PAX8 expression levels of the single clones, confirming the role of PAX8 as oncogene *in vivo*. Our data provide the first evidence of a clear involvement of PAX8 in the *in vivo* tumorigenesis of ovarian cancer cells.

Recent evidences showed that high-grade serous carcinoma (HGSC) originates in the Fallopian tubal secretory epithelial cells, which are positive for PAX8 expression (Perets et al. 2013), thus prompting us to see if a subset of PAX8 target genes is relevant for cancer initiation or maintenance by identifying novel PAX8 targets both in ovarian cancer and Fallopian tube cells. In order to define the transcriptional networks functionally regulated by PAX8 as well as to identify its direct and indirect targets, we performed transient RNA interference to knockdown PAX8 gene expression in FT-194 and SKOV-3 cells and we analyzed the gene expression profile by RNA sequencing (RNA-seq). The results obtained suggest that PAX8 regulates several pathways, mainly involved in the regulation of reproductive system disease, cellular growth and proliferation, cell death and survival, and cellular movement. We have chosen the commons genes from both the FT-194 and SKOV-3 cells line and predicted affinity of their 5'-flanking regions to PAX8 binding. We identified potential PAX8 targets based on the presence of PAX8 binding site(s) in their 5'-flanking sequences and tissue-specificity. Our results show that PAX8 is involved in the regulation of a large number of genes in both Fallopian tube cells and ovarian cancer cells that will need to be further evaluated to fully ascertain PAX8 direct targets.

6. CONCLUSIONS

Overall, the findings of our study definitely suggest a key role of PAX8 in the progression of ovarian cancer. Our results clearly indicate that PAX8 interfering is able to reduce cell growth and survival as well as cellular motility and invasion of ovarian cancer cells. Although further studies are needed, some PAX8 target genes have been identified.

In conclusion, we believe that the role of PAX8 as a potential diagnostic marker and therapeutic target for ovarian cancer represents an exciting research area that promises to deliver new insights into the onset and growth of such aggressive cancers.

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