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"PAX8 in ovarian carcinoma: identification of new downstream networks and target genes"

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ABSTRACT

PAX8 is a transcription factor involved in the tissue-specific expression of several genes during development, tissue homeostasis and cancer. Recently, PAX8 has been reported to be an important marker for the diagnosis of ovarian carcinoma with a pivotal function in the tumorigenic phenotype of ovarian cancer cells. PAX8 is normally expressed in Fallopian tube secretory cells but not in ovarian surface epithelial cells; however, its expression is detected in the majority of high-grade serous ovarian carcinoma (HGSC) supporting the tubal origin of this cancer. To determine whether PAX8 contributes to ovarian cancer development, we initially conducted a transcriptome analyses to determine the distinctive molecular profiles of the Fallopian tube epithelial secretory cell line (FT194) and the ovarian cancer cell line (SKOV3), before and after PAX8 silencing. The bioinformatics analysis revealed several GO categories enriched in both PAX8-silenced FT-194 and SKOV3 cells. Among those categories, the results showed that both "cell migration" and the "positive regulation of cell migration" bioprocess displayed transcriptional change of 5% in SKOV3 cells and the adhesion category shows change of about 16% in SKOV3 and 14% in FT-194 cells. With respect to specific pathways, the highest differential changes upon PAX8 silencing were found in angiogenesis, Wnt, cadherin and integrin signalling pathways, in both cell types.

Since migration and adhesion are important biological processes in both physiological and pathological conditions, migration and adhesion assays were performed using a primary human fallopian tube secretory cells (Primary hFTSECs) and a panel of ovarian cancer cell lines (SKOV3, KURAMOCHI, OVSAHO and PEA1). Interestingly, our results show that inhibition of PAX8 expression in Primary hFTSEC and in epithelial ovarian cancer cell lines significantly reduces the ability of the cells to migrate and adhere on Fibronectin and/or Collagen I substrates.

Integrins are reported to be the major regulators of cellular attachment with the extracellular matrix and are required for cellular migration. In our transcriptome analysis, Integrin β_3 was significantly downregulated after PAX8 silencing in SKOV3 cells. Therefore, we performed qRT-PCR

on Primary hFTSEC and our panel of ovarian cancer cell lines and the results show a strong reduction of Integrin β_3 expression in all ovarian cancer cell lines after PAX8 silencing, respect to the control cells. In parallel, we also show that loss of PAX8 does not affect the expression of Integrin αv, the ligand of Integrin β_3 involved in ovarian cancer tumorigenesis. The Immunofluorescences assays of the functional heterodimer $\alpha_v\beta_3$ was tested in Primary hFTSEC and KURAMOCHI cell lines and in PAX8 silenced cells the signal was decreased. In conclusion, we believe that it is of great relevance to further study and decipher the link between PAX8 and Integrin β_3 because it could help uncover the role of PAX8 in HGSC development.

1. INTRODUCTION

1.1 Ovarian cancer

Ovarian cancer (OC) is a generic term for a constellation of heterogeneous groups of cancer that involve the ovary. Around 240,000 new cases of OC are diagnosed each year making it the seventh most common cancer and the leading cause for all gynecological cancer deaths worldwide. It is also the fifth leading cause of death in women with around 152,000 cases every year (Ferlay et al. 2015). Moreover, compared to many other cancers that have witnessed a significant decrease in mortality, the death rate of ovarian cancer has remained constant over the past 40 years with a 5-year survival rate of less than 30% (Siegel et al. 2015).

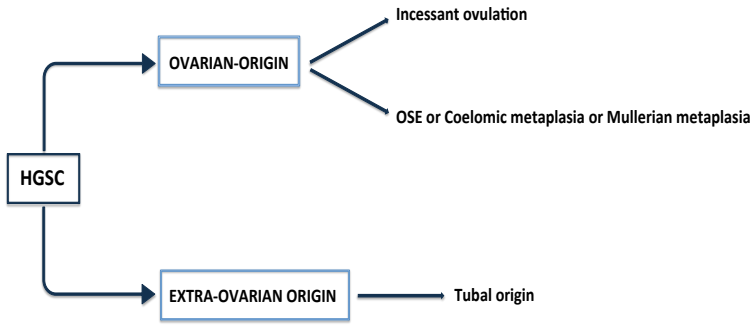
Based on the cell-of-origin, OC is divided into different types of tumors: epithelial (95%), germ line (3%), and sex-cord stroma (2%). Amongst these, the most common and lethal is the Epithelial Ovarian Cancer (EOC) type that is further divided into five histological subtypes: high-grade serous carcinoma (HGSC, 68%), endometroid carcinoma (EMC, 20%) clear-cell carcinoma (CCC, 4%), mucinous carcinoma (MC, 3%) and low-grade serous carcinoma (LGSC less than 5%). This classification is based on the pattern of differentiation and tumor cells morphology (Elisabetta Kuhn et al. 2011).

After extensive histopathological and molecular study of various types of ovarian cancer, Kurman et al in 2016 proposed a new dualistic model of epithelial ovarian carcinogenesis. According to this new model the EOC is divided into two main groups: type I and type II tumors. Type I tumors include endometriosis-related tumors such as endometrioid, clear cell and seromucinous carcinomas; the low-grade serous carcinomas; mucinous carcinomas and malignant Brenner tumors. These types of tumor develop from benign extraovarian lesions that, after implanting on the ovarian surface, undergo a malignant transformation. They usually grow slowly and are genetically stable with characteristic gene signatures such as mutations in KRAS, BRAF, PTEN and CTNNB1. They are often diagnosed at an early stage when they confine to the ovaries and usually have a good prognosis, representing only 10% of the deaths from ovarian cancers (Kurman and Shih 2016).

Type II tumors are composed for the most part, of high-grade serous carcinomas that can be further subdivided into different subtypes. Many type II carcinomas develop from fallopian tube epithelial carcinomas that subsequently involve the ovaries and extra-ovarian sites. They are genetically unstable with several characteristic gene signatures such as TP53 mutations, BRCA1 loss and PIK3CA mutations. They are characterized by drastic development with high proliferative and metastatic features. In >75% of cases the disease is detected in advance stages, usually showing an intra-abdominal spread, frequently accompanied by ascites. Unfortunately, the Type II tumors are the most common, with very poor 5-year survival rate, causing 90% of all ovarian cancer deaths (Kurman and Shih 2016).

1.1.1 Theories about the origin of HGSC

Most cancers involving the ovaries are called “Ovarian Cancer” but many of them may not originate in the ovaries. The incomplete understanding of the origin and pathogenesis of each type of ovarian cancer may impede the development of prevention, early detection and treatment methods. The HGSC causes 80% of all malignant ovarian fatalities (Bowtell 2016). The low 5-year survival rate is mainly due to the loss of specific symptoms in the early stages of the disease and consequently the diagnosis is usually after the metastasis. The exact process of the development of HGSC is still unclear. Therefore, for a better comprehension of this disease it is necessary to study and understand the process of its origin. Although, there have been several theories proposed to explain the nature and the development of the HGSC. There are mainly two schools of thought based on the primary site of the HGSC: the ovarian origin and the extra ovarian origin.



Originally, the primary site of HGSC was attributed to the ovary and in particular, the ovarian surface epithelium (OSE). The "incessant ovulation" hypothesis is the one of the most popular theories according to which the ovarian surface epithelium is transformed because of repetitive injury to the OSE during each ovulatory cycle. This repetitive injury causes changes in hormone levels and an increased inflammation, leading to DNA damage and OSE transformation that further develops into HGSC. Consequently, the risk for the transformation process increases with higher number of ovulations and therefore, oral contraceptives that reduce ovulation, are associated with lesser risk of ovarian cancer (Fathalla 2013).

The other theory that supports the ovarian origin of HGSC is the “OSE or coelomic metaplasia”. According to this theory, incessant ovulation and each cycle of repair drive a metaplastic change towards a more Mullerian-type epithelium. This theory suggests that to undergo metaplasia, the cells retain their primitive stem-cell like pluripotent nature. The transformed cells exhibited the ability to acquire characteristics similar to that of fibroblasts and also expressed stem-cell markers like NANOG (Auersperg 2013).

Between 1990 and 2000, the theories that supported the ovarian origin of HGSC began to falter. During this period, it was observed that BRCA1 and BRCA2 mutations were associated with increased risk for ovarian cancer. Therefore, women with these germ line mutation were suggested to undergo prophylactic tubectomy and oophorectomy (Olivier et al. 2004; Walker et al. 2015). Surprisingly, while looking for some early indicators of ovarian cancer, several hidden lesions were found in the fallopian tubes fimbria, which were later named as serous tubal

intraepithelial carcinoma (STIC) (Zweemer et al. 2000; Carcangiu et al. 2004). It is important to note that these samples did not show any ovarian serous carcinoma (Medeiros et al. 2006; Shaw et al. 2009). More importantly, while comparing the gene expression profiles of HGSC, FTE and OSE, HGSC and Fallopian tube secretory epithelial cells, showed more similarities than HGSC and OSE. In particular, they revealed that most of the STIC lesions harbor the same TP53 mutation as the concurrent HGSC, indicating their possible clonal nature (Kuhn et al. 2012). The hypothesis that the fallopian tube is the primary site of most high-grade serous carcinomas start to be supported by several research findings. Thus, a new school of thought that supports the extraovarian origin of the HGSC was developed. It was proposed that for the transformation process of secretory epithelial cells of the Fallopian tube TP53 should be mutated and acquire a p53 signature (Kuhn et al. 2012). These cells further accumulate mutations induced by hormones that further promote the development of non-invasive Serous Tubal Intraepithelial Carcinoma (STIC) in the Fallopian tube. The proximity to the ovary and the paracrine factors secreted by it, possibly induces further neoplastic changes. These STICs, on reaching the adjoining the OSE surface, become fully metastatic and aggressive HGSC. The Figure 1 shows the classical process of development from the normal Fallopian Tube epithelium (FTE) to the highly invasive HGSC, through accumulation of p53 signature and development of STIC. Designing mouse models for HGSC has been difficult due to the genetic complexity of the disease. In a recent report, Zhai et al showed a new mouse model for ovarian cancer that uses Ovgp1 driven Cre as a FT-specific promoter to inactivate Brca1, Trp53, Rb1, Nf1 and Pten. The loss of the expression of these specific genes in the mouse FT epithelium mimics the tumorigenic development of human HGSC. This evidence along with others, seem to suggest that the majority of the HGSC possibly originate as STICs in the FTE (Kim et al. 2012; Zhai et al. 2017). Recently it has been reported that PAX8 is expressed in the Fallopian tube secretory epithelial cells and its expression is retained in the HGSC cells supporting the tubal carcinogenesis hypothesis.

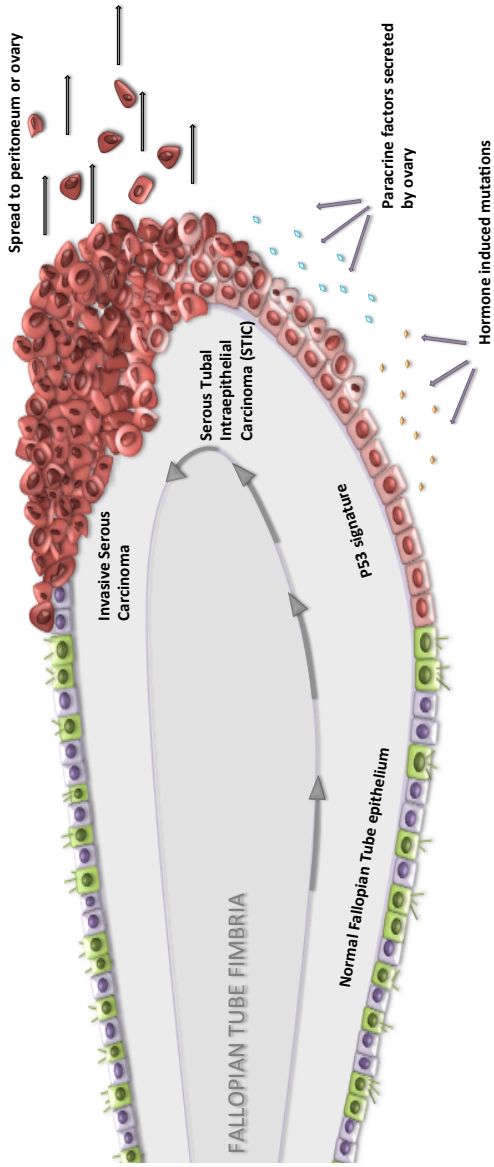


Fig. 1 Transformation process of secretory epithelial cells of Fallopian tube fimbria. Following TP53 mutations, the cells further accumulate hormone-induced mutations that promote the development of non-invasive STIC. The proximity to the ovary and possibly the paracrine factors secreted by it induces further neoplastic changes becoming fully metastatic and aggressive HGSC.

1.2 PAX gene family

The PAired boX gene family (PAX) is a group of transcription factors that are well known for their role during embryogenesis. This family is made up of nine members (PAX1-9) and their expression is strictly regulated in both temporally and spatially, critical for normal embryonic development (Mansouri et al. 1996). PAX genes have been described for the first time in the Drosophila model and their expression is evolutionarily well conserved in many species including humans, mice, zebrafish, birds, frogs, flies and worms (Bopp et al. 1986; Dahl 2005). The PAX gene family is named after the discovery of the paired-box DNA binding domain that is common for all the members. On the basis of their structure, the PAX genes are further divided into four subgroups (I-IV) based on the differences in two of the three regions: Paired box (common in all members), Octapeptide (presence or absence) and Homeodomain (presence, absence or truncation) (Treisman et al. 1991) (Fig.2).

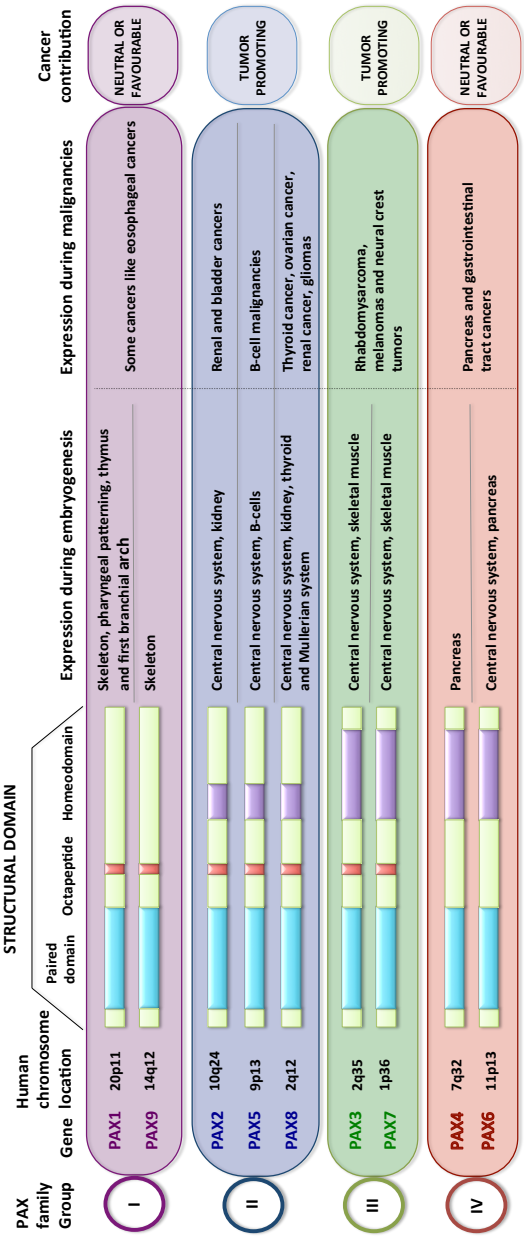


Fig.2 Structural domains, expression during embryogenesis and malignancies, and cancer contributions of PAX family members subgroup.

The Paired box domain is highly sequence specific in its DNA binding region, it is 128 amino acid long and located at the amino-terminal end of the protein. The DNA–binding paired-box domain is made up of two sub-domains named PAI and RED. The entire PAIRED domain is involved in its interaction with DNA and in particular, the N-terminal PAI sub-domain interacts directly with the DNA and the C-terminal RED sub-domain indirectly (Treisman et al. 1991).

In addition to the Paired domain, the homeodomain can also interact with the DNA. This structural domain can be, in certain PAX genes, additional or partial. PAX3, PAX4, PAX6 and PAX7 have a three-helix homeodomain while PAX2, PAX5 and PAX8 have a partial one-helix homeodomain. Generally, homeodomains assist in sequence specific binding to the DNA, and, particularly, the homeodomain of the PAX proteins recognize the palindromic sequence TAAT(N)2–3ATTA (Treisman et al. 1991; Mayran et al. 2015).

The octapeptide is an eight amino-acid domain present in all PAX proteins, except for PAX4 and PAX6. It is highly conserved and functions as a transcriptional inhibitory motif. The direct interaction of the octapeptide domain is functionally important for its repressive activity and, for example, the interaction of the octapepdtide domain of PAX protein is functionally important, as this repressive activity is associated with dysregulation of the Wnt pathway that is attributed to several cancers (Mayran et al. 2015).

The PAX gene expression is critical during the embryogenic development and their roles are well conserved across species. Their expression is generally observed until the organogenesis and may persist only in a few specific organs in the adult. Their presence is attributed to the regulation of cell fate decisions and has been associated with enhanced cell-proliferation, repression of apoptosis, inhibition of terminal differentiation and promotion of stem cell features (Muratovska et al. 2003). However, in the last decade, their aberrant expression is increasingly associated with several malignancies and other pathologies. Despite this association, their precise role in tumor progression is still obscure and it has been reported that their overexpression or aberrant expression does not seem to be sufficient to cause malignancy (Muratovska et al. 2003). During organogenesis, PAX1 and PAX9 are well-characterized for their role in skeleton, pharyngeal patterning, development of thymus and first branchial arch; PAX2, PAX3, PAX5, PAX6, PAX7 and PAX8 in central

nervous system; PAX2 and PAX8 in kidney; PAX5 in B-cells; PAX8 in thyroid and Mullerian system; PAX4 and PAX6 in pancreas; and PAX3 and PAX7 in skeletal muscle (Lang et al. 2007; Blake and Ziman 2014).
In malignancies and other pathogenesis, it has been reported that PAX genes such as PAX2, PAX3, PAX5, PAX7 and PAX8 have tumor promoting role with an anti-apoptotic, pro-proliferative and pro-metastatic effect (Fig. 3); the other PAX genes such as PAX1, PAX4, PAX6 and PAX9 have not been reported to have tumor enhancing functions. Among the PAX genes with tumor promoting features, PAX3 and PAX7 are involved in sarcomas, specifically Rhabdomyosarcoma, melanomas and neural crest tumors (Relaix et al. 2004) PAX5 in several B-cell malignancies (Busslinger 2004); PAX2 in several renal and bladder cancers (Muratovska et al. 2003); PAX4 and PAX6 are implicated in the cancers of the pancreas and gastrointestinal tract (Wang et al. 2008); PAX8 is involved in thyroid cancers, ovarian cancers, renal cancers, gliomas (Tacha et al. 2011). Contrarily, with respect to PAX genes correlated with favorable prognosis, PAX1 and PAX9 are involved in eosophageal cancers (Gerber et al. 2002) (Fig. 2).
The PAX genes are also involved in cancers by producing a fusion oncopeptide that is constitutively expressed through chromosomal translocations. The best examples are PAX3-FKHR, PAX7-FKHR in alveolar rhabdomyosarcoma (Gerber et al. 2002), PAX5 – IGH in Non-Hodgkins Lymphoma (Souabni et al. 2007), PAX8-PPAR in Follicular thyroid carcinoma (Sugg et al. 1998). It is important to note that PAX8 exhibits role in major hallmarks for metastasis such as inhibiting cell death, propagating self-renewal and in Epithelial Mesenchymal Transition (Di Palma et al. 2014).

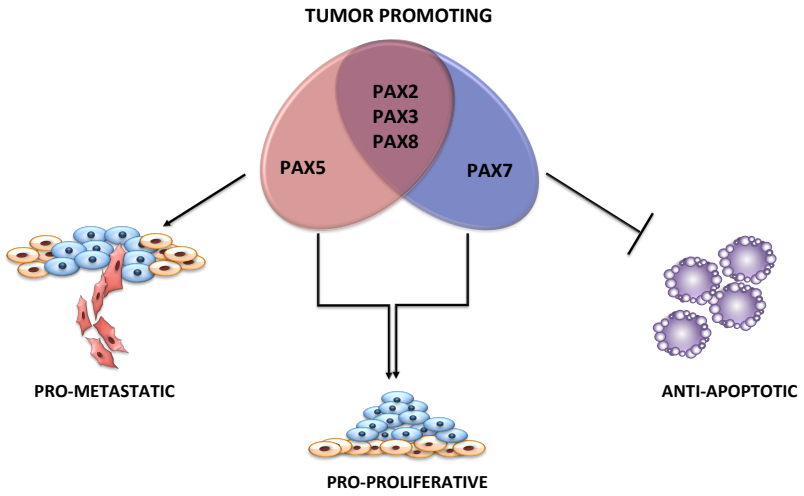


Fig.3 Overview of the roles of PAX genes involved in tumor promoting processes.

1.3 The transcription factor PAX8

PAX8 is a transcription factor that was first isolated in 1990 in mice and then in 1992 in humans (Poleev et al. 1992). PAX8 is now well known for its important role in the cell fate determination and development of several organs like thyroid, kidney, eyes, inner ear, brain and Müllerian tract (Gerber et al. 2002). Like the other PAX in the subgroup II, PAX8 is composed of Paired domain, an octapeptide and a truncated homeodomain. The gene that codes for the human PAX8 protein is present in Chromosome 2 at position 2q12-1447 and it is composed of 12 exons. The translation begins from exon 2 that has the start codon. The exons 3 and 4 encoded for the Paired-box domain that is present in the amino terminal region of the protein. Exon 5 codes for the octapeptide while exons 7 form the truncated transactivating homeodomain (Yusuf et al. 2012).

PAX8 has five different isoforms, namely, PAX8A, B, C, D and E, from different RNA transcripts produced as a result of alternative splicing of 8-10 exons.

PAX8A, which is composed of 450aa, is the longest and the most

common isoform and includes all the codons from exon 2 to 12. PAX8B does not have exon 9. PAX8A and PAX8B, each have exon 10-11 with unique serine, threonine and tyrosine rich transcriptional activation domain unlike other isoforms. PAX8C has a shorted exon 9 as it utilizes an internal exon 9 5'-splice site and due to this, it has an altered reading frame producing the stop codon in exon 11 thereby having a shorter proline-rich carboxyl-terminal. PAX8D has lacks exon 8 and 9, while PAX8E, has exons 8-10 deleted. Both PAX8D and PAX8E have reading frame identical to PAX8C and produce truncated proteins. The transcriptional activity is higher in PAX8A and PAX8B as compared to PAX8C (Kozmik et al. 1993). Very little is known about the post-transcriptional modifications of the PAX8 protein. Since there are serine and threonine sites in the domain of the PAX8 protein, there could be phosphorylated with the possible involvement of PKA (Protein Kinase A) (Poleev et al. 1997). However, the exact sites for phosphorylation are yet to be defined.

In thyroid, PAX8 has been known to undergo sumoylation by the conjugation of SUMO at lysin residue 309 thereby stabilizing the protein by preventing its degradation (de Cristofaro et al. 2009).

Most of the transcriptional role of PAX8 is known in thyroid context and it is involved in several pathways that contribute to carcinogenesis. Retinoblastoma (RB), a tumor suppressor, is a known positive transcriptional co-activator of PAX8 and interacts with its partial homeodomain (Miccadei et al. 2005). PAX8 also is involved with RB in a reciprocal relation wherein PAX8 regulates E2F1 promotor and stabilizes RB helping in tumor cell growth. PAX8 is also known to promote tumor cell survival by suppressing the expression of another well-known tumor supressor, TP53 through TP53inp1 (Di Palma et al. 2013). Wilms Tumor 1 (WT1), another tumor suppressor was reported to have transcriptionally activated by PAX8 (Siehl et al. 2003). PAX8 is also reported to be involved in the activation of Bcl2, an important anti-apoptotic protein56. Transforming Growth Factor–b1 (TGFB1), whose role is implicated in the favoring the tumor microenvironment, is also reported to control PAX8 transcription (Hewitt SM et al. 1997).

In addition, PAX8 is implicated in an oncogenic rearrangement in thyroid carcinomas caused by a translocation between chromosomal regions 2q13 and 3p25. This rearrangement results in a fusion transcript wherein most of the coding sequence of PAX8 (2q13) is fused in frame with the entire

coding exons of PPARγ1 (3p25). The PAX8 promoter is highly active in thyroid follicular cells and drives the expression of the fusion transcript, resulting in high level expression of the fusion transcript and protein PPFP (PAX8-PPARγ fusion protein). Although the specific mechanism of PPFP action is yet to be defined, it is known that PPFP has the DNA binding domains of both PAX8 and PPARγ. Therefore, a plausible mechanism of oncogenesis is the modulation of the downstream pathways of PAX8 or PPARγ (Priyadarshini and Ronald 2015). Thus, PAX8 though primarily known as a differentiation promoting transcription factor has been in different contexts reported to aid in tumor progression and maintenance.

1.3.1 PAX8 expression in normal tissues

PAX8 is an important transcription factor involved in the development of various organs such as thyroid, kidney, Müllerian tract, vertebral column, hindbrain, eye and inner ear (Blake and Ziman 2014). It is also necessary for the maintenance and has been shown to be a lineage-specific marker of organs like thyroid, kidney and the Müllerian tract (Tacha et al. 2011).

Because of the high sequence homology between PAX8 and the other members of the subgroup II (PAX2 and PAX5), some reports of PAX8 staining in lymph nodes, pancreas, and neuroendocrine cells of stomach and colon cannot be considered as a fact. This is attributed to the cross-reactivity of PAX8 polyclonal antibody used and not because of PAX8 expression in these tissues. To resolve this issue, a monoclonal PAX8 antibody with high specificity was used to identify PAX8 positive tissue (Toriyama et al. 2014).

Consistent with the role of PAX8 as a lineage-specific marker, it has been shown to be express in normal adult tissues of thyroid, kidney and the Müllerian tract. Its expression has been demonstrated in the developing thyroid gland (Damante G, Tell G 2001) and it efficiently determines the differentiated phenotype typically seen in the adult follicular thyroid cells (Pasca di Magliano et al. 2000).

In kidney, its staining was detected in the normal tissue with focal segmental staining of glomerular parietal epithelial cells and diffuse staining of collecting duct epithelial cells (Poleev et al. 1992). In the male

genital tract, seminal vesicles and epididymis were diffusely positive, but not germ cells like, Leydig cells or Sertoli cells (Ozcan et al. 2011).

With respect to the Müllerian duct, PAX8 is detected in the embryo during the organogenesis and development of the Müllerian duct. Moreover, PAX8 is said to be involved in the formation of the epithelial layer (Kobayashi 2004). It is retained throughout the formation of the fallopian tube and uterine epithelium. In the female genital tract, strong and diffuse PAX8 staining was observed in the epithelial cells of the endocervix and the endometrium. When the epithelial layer of the fallopian tube differentiates into ciliated and secretory cells, PAX8 stained only basal and secretory cells where its expression is retained and absent in ciliated cells (Bowen et al. 2007).

1.3.2 PAX8 in cancer tissues

The expression of PAX8 in neoplastic tissues is well studied in several cancers by analysing the tissue expression profiles of cancer patients. PAX8 is observed in carcinomas of ovary, uterus, kidney, prostrate, gliomas, Wilms' tumor and Kaposi sarcoma. The first report of PAX8 in malignancy was in Wilms' tumor in 1992 (Poleev et al. 1992). The expression of PAX8 was then reported in several human thyroid neoplasms (Belfiore et al. 1994). Several reports of PAX8 expression in cancers such as renal neoplasm, ovarian carcinomas, gliomas were described establishing a definite correlation between PAX8 and several epithelial malignancies (Hunter et al. 2015). Further, PAX8 is now known to be a useful immunohistological marker that helps differentiate Mullerian from non-Mullerian tumors (Tavanafar and Heidarpour 2014).

Tacha et al. examined the immuno-histochemical expression of PAX8 in multiple normal and neoplastic tissues. Renal cell carcinomas tested positive for PAX8 in 90% of the cases, ovarian cancers for 79% of the cases and thyroid cancer for 90% of all cases (Tacha et al. 2011). The *PAX8/PPARG* gene fusion, that was previously mentioned, was found in 30–35% of follicular thyroid carcinomas and in a substantially smaller fraction of follicular variant papillary thyroid carcinomas. This rearrangement is very occasionally found in follicular adenomas (Priyadarshini and Ronald 2015).

In endometrial cancers, 84% of the cases were positive for PAX8 expression and in cervical cancers, PAX8 was observed in 83% cervical adenocarcinomas whereas 98% squamous cell carcinomas cases were negative for the protein expression. In bladder cancers, PAX8 was negative in 93% of all the cases including all bladder adenocarcinomas. PAX8 expression was observed in only one case of lung cancer (99% negative) and was 100% negative in cancers of the colon, breast, prostate, liver, testicular, stomach, esophagus, melanoma, gastrointestinal stromal tumors, leiomyosarcoma, and pheochromocytoma (Tacha et al. 2011).

Laury et al. conducted a study in which PAX8 immunohistochemistry was performed on 1357 tumors (486 tumors in whole-tissue sections and 871 tumors in tissue microarrays, predominantly epithelial) from multiple organs. Nuclear PAX8 staining was present in 91% of thyroid tumors, 90% of renal cell carcinomas (RCCs), 81% of renal oncocytomas, 91% of cervical epithelial lesions, and 98% of endometrial adenocarcinomas. The remaining tumors, such as those from the prostate, colon, stomach, liver, adrenal gland, head and neck, small cell carcinomas from the lung were PAX8 negative (Tacha et al. 2011).

PAX8 expression and its association with ovarian epithelial cancers deserves a special focus as its presence in most of the tissues is well replicated and documented in several independent studies apart from the ones mentioned above. Laury et al. reported that PAX8 staining was present in 99% (164 of 165) of high-grade serous ovarian carcinomas, 71% (32 of 49) of nonserous ovarian epithelial neoplasms, and all (100%) low-grade ovarian carcinomas and serous borderline tumors. It is important to mention that strong PAX8 staining was highly specific for ovarian serous tumors according to both Laury and Tacha et al. (Laury et al. 2011; Tacha et al. 2011)

Recently, Hong-Juan Chai et al. demonstrated that PAX8 was highly expressed in primary epithelial ovarian cancer (PEOC) with an overall 92% positivity. In addition, their study revealed that PAX8 expression level was associated with the degree of cancer cell differentiation, FIGO stage, and survival rate, indicating that PAX8 is a potential marker for the diagnosis of PEOC (Chai et al. 2017). This could be relevant in the therapeutic approach. Apart from the ones mentioned above, there are various recent reports that vouch for the specificity of PAX8 expression specifically in HGSC. Due to this PAX8 has become a reliable

immunohistological marker to identify and diagnose HGSC.

Figure 4 highlights the role of PAX8 in organogenesis, expression in adult tissue in both normal and neoplastic conditions. If one observes closely, there seems to be a definite association between expression of PAX8 in normalcy and malignancy, as in many transcriptional factors that are involved in in development and cancers.

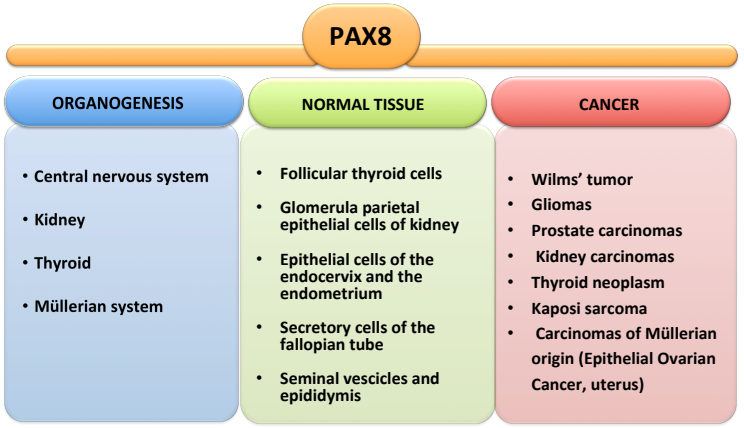


Fig.4 Expression of PAX8 during the organogenesis, in adult tissue and cancers.

1.3.3 Pathways regulated by PAX8 in ovarian cancer

To have better insights regarding the possible underlying mechanisms by which PAX8, that has a normal expression in Fallopian tube is aberrantly retained in metastatic ovarian cancers like HGSC, several groups studied the gene expression analysis before and after PAX8 silencing in Fallopian tube cell-line and ovarian cancer cell line.

In 2016, Elias et al, published their findings of how the epigenetic changes that govern the PAX8 binding sites are modified between fallopian tube and ovarian cancer (Elias et al. 2016). They studied the PAX8 cistrome by comparing three fallopian tube cell-lines (FT33, FT194 and FT246) and three high-grade serous cancer cell lines (KURAMOCHI, OVSAHO and JHOS4) before and after PAX8 silencing. Cistrome is defined as the genome wide map of the binding

sites of a transcription factor. Their study showed that between fallopian tube and the ovarian cancer cell-lines, the ovarian cancer cells had significantly reprogrammed their PAX8 cistrome. Further on analysing by RNA-seq and ChIP-Seq, the genes that are differentially expressed between these two cell types were located and clustered around the PAX8 binding sites. Additionally, on careful scrutiny of the PAX8 cistrome alterations, there seems to be an increase in the interactions between PAX8 and TEAD. This suggests that the Hippo-YAP signalling pathway, which is known for their important role in many cancers, could interact with PAX8 and be an important mediator to regulate transformation in ovarian cancer. Their group further investigated this relation and proposed that PAX8 functions differentially upon transformation and thereby promote cancer progression through Hippo-YAP pathway (Elias et al. 2016).

To highlight the significance in exploring the PAX8 pathway in epithelial ovarian cancer, Kar et al. in 2017 showed that of loci that are susceptible to alterations in Serous Epithelial Ovarian Cancers (SOC), the putative PAX8 target genes with binding sites for PAX8 were enriched (Kar et al. 2017). This is the first study that distinguishes binding sites for PAX8 as the governing change amongst SNP (Single Nucleotide Polymorphisms) that are enriched in epithelial ovarian cancer and increases its risk, much like Estrogen receptors in breast cancer or androgen receptors in prostate cancer. This study analysed the 615 Transcription factor (TF)- target gene sets from the two largest and available Serous Ovarian Cancer risk Genome-wide Association Studies data sets. They concluded that the PAX8 targets were the most altered amongst the gene sets. Their study indicated how the SOC risk was driven by a network of PAX8 and 15 select genes such as BNC2 and HOXB7. These genes were further validated for their binding to PAX8 by ChIP-seq analysis (Kar et al. 2017).

Along the same line, Adler et al, also in 2017 investigated PAX8 cistrome in epithelial ovarian cancer (Adler et al. 2017). This report along with others demonstrated the decrease in anchorage dependent and independent growth in ovarian cancer cell lines (HeyA8 and IGROV1) upon PAX8 silencing. PAX8 cistrome in these cell-lines were characterized using ChIP-seq along with ChIP-seq for acetylated histone subunit 3 (H3K27ac) to analyze PAX8 binding sites in active chromatin. The two cell lines showed difference in the number of PAX8 binding sites corresponding with active chromatin, approximately 60% in IGROV1 and

25% in HeyA8 cells. The enormous difference could be attributed to the difference in histotype of the cancer having isolated from different patients. This study also characterized the PAX8 binding motif and identified candidate PAX8 co-regulators and target genes. They concluded that PAX8 binding was enriched at superenhancers and controlled genes that encompass differentiation, development and tumorigenesis. They reported that amongst the many developmental pathways that were augmented in the PAX8 cistrome, tissue morphogenesis, apoptosis, EMT, Notch signalling were notable. This study indicates the direct and indirect regulatory gene targets of PAX8 throughout the genome (Adler et al. 2017).

More recently, Ghannam-Shahbari et al. in a study published in 2018 have reported that PAX8 can directly bind to the first intron-exon boundary of TP53 in Fallopian tube and also to mutant TP53 in HGSC. The PAX8 activated mutant TP53 further causes a cytoplasmic p21 accumulation resulting in a proliferative effect observed in HGSC (Ghannam-Shahbari et al. 2018). It is more than plausible that PAX8 could have other pathways to execute its pro-oncogenic roles in HGSC. However, from the above studies, one can consider PAX8 as a putative and an important regulator of HGSC. This also indicates that inhibition of the PAX8 pathway or many of its downstream regulators in a combinatorial method could be a possible therapeutic methodology to contain HGSC (Ghannam-Shahbari et al. 2018).

2. AIM OF THE THESIS

In this thesis, I will discuss the role of the transcription factor PAX8 in ovarian carcinoma through the identification of its new putative target genes and downstream networks. Particularly, I will address the two pivotal aims of my Ph.D. project: 1) identification of PAX8 downstream pathways in Fallopian tube secretory cells (FT194) and epithelial ovarian cancer cells (SKOV3), by means of the RNA-seq strategy; 2) analysis of migration and adhesion properties of primary human fallopian tube secretory cells (Primary hFTSECs), SKOV3 and other HGSC cell lines following PAX8 silencing focusing on the possibility that Integrin β_3 could be involved in the modulation of these two processes.

Concerning the first aim, the molecular mechanism(s) by which PAX8 fosters High-grade serous carcinoma (HGSC) is still unclear. To determine how PAX8 might contribute to ovarian cancer development, we initially conducted a transcriptome analyses to determine the distinctive molecular profiles of the Fallopian tube secretory cells and ovarian cancer cells (FT194 and SKOV3 cell lines, respectively), before and after PAX8 silencing. Through a bioinformatics analysis, we examined several GO categories and pathways enriched in both PAX8-silenced FT194 and SKOV3 cells.

As to the second aim, based on the results obtained by RNA-seq analysis, we analyzed the effect of PAX8 silencing on migration and adhesion properties of Primary hFTSECs, SKOV3, KURAMOCHI, OVSAHO and PEA1 cells. The goal was to understand if these two important processes of cancer progression are affected by PAX8. In the last part of my study, I focused on Integrin β_3 that is an adhesion molecule involved in both migration and adhesion ability of ovarian cancer cells, probably downstream of PAX8.

3. MATERIALS AND METHODS

3.1 Cell culture

Immortalized Fallopian tube secretory epithelial cell line FT194 was provided by Dr. R. Drapkin (Boston, USA). This cell line was grown in DMEM-F12 medium (Euroclone) supplemented with 2% Ultroser G serum (PALL) and 1% penicillin/streptomycin.

The human ovarian cancer cell line SKOV3 was provided by the CEINGE Cell Culture Facility (Naples, Italy). High-grade serous ovarian cancer cell lines KURAMOCHI and OVSAHO were obtained from the Japanese Collection of Research Bioresources Cell Bank (JCRB). All these cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Euroclone).

The human ovarian cancer cell lines PEA1 and PEO14 were purchased from Sigma-Aldrich and were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 2 mM sodium pyruvate and 1% penicillin/streptomycin (Euroclone).

The ovarian surface epithelial cancer cell line OVCAR-3 was obtained from ATCC and was maintained in RPMI-1640 medium supplemented with 20% fetal bovine serum, 0.01mg/ml bovine insulin and 1% penicillin/streptomycin (Euroclone).

3.2 Tissue samples

Fresh FT fimbria specimens were obtained from the Department of Gynecology of the AOU “Federico II” Hospital of Naples, Italy, with approval of the institutional review board. The fimbrial tissues used in this study are collected from surgical procedures for benign gynaecological indications. Specifically, cases of inflammatory disease, infection, and extensive adhesions were excluded.

3.3 Primary human Fallopian tube secretory epithelium ex-vivo culture system

The FT tissue was wash several time in 0.9 % NaCl solution and 1% penicillin/streptomycin until traces of blood was completely removed. To help the stabilization of the tissue, it was washed in CHANG MEDIUM C (IrvineScientific). The dissection was performed with sterilized scalpel and needle by cutting the tissue into very small 1mm sized pieces. The dissected tissue were incubated for O/N at 37°C for enzymatic digestion with 0.8 mg/ml of collagenase I (Sigma C9407-100MG) on 60 mm culture dishes supplemented with DMEM-F12 (Euroclene) with reduced serum 5% fetal bovine serum (Euroclone) and 1% penicillin/streptomycin (Euroclone). Supernatant collected After O/N digestion was centrifuges at 1200 rpm for 5 min and the pellet composed of dissociated cells was plated in collagen coated 60 mm plate supplemented with DMEM-F12 (Euroclone), 2% Ultroser G serum (PALL) and 1% penicillin/streptomycin (Euroclone) media. After a visual estimation of cells their grew in plates, epithelial-line cells that grew in clusters were carefully trypsinised using clonal cylinders and replated onto a fresh 60 mm collagen coated culture plate. These cells, named Primary hFTSECs, were analysed for Fallopian tube secretory epithelial cell markers such as PAX8 and OVGP1 using Immunofluorescence. At every passage, the cells were verified for these markers and only them used for experiments. The isolation of tissues was done using the help of Dr. Antonella Izzo at the Prenatal Cytogenetics and Diagnostic Services at the University of Federico II, Naples, Italy.

3.4 Cell culture transfection

In all the experiments, PAX8 expression was transiently downregulated by means of RNA interference.

For the RNA-seq experiments SKOV3 and FT194 cells were plated in triplicates at a concentration of 2×10^5 cells/60-mm tissue culture dish and were transfected after 24 hrs with 5 nM PAX8 siRNA (Ambion, Life Techonologies, siRNA ID s15403) or siRNA Non Targeting (Ambion, Life Technologies, siRNA ID 4390843) as scramble, using the

Lipofectamine RNAiMAX transfection reagent (Invitrogen) following the manufacturer's protocol. Cells were collected 24 hrs after transfection.

For the qRT-PCR validation experiments SKOV3 and FT194 cells were harvested at 24 hrs, 48 hrs and 72 hrs after transfection and PEA1, PEO14 and OVCAR-3 after 24 hrs.

For the migration and adhesion assays Primary hFTSEC, SKOV3, KURAMOCHI, OVSAHO and PEA1 cell lines were transfected with 5 nM PAX8 siRNA (Ambion, Life Techonologies, siRNA ID s15403) or siRNA Non Targeting (Ambion, Life Technologies, siRNA ID 4390843) as scramble and harvested 48 hrs after transfection.

3.5 RNA, cDNA and qRT–PCR

Total RNA was extracted using the RNeasy Mini kit (Qiagen). Human Fallopian tube RNA was purchased from Origene (CR559726). The cDNA was synthesized using the iScript cDNA Synthesis kit (BIORAD, Hercules, CA). Real-time qPCR analysis was performed using the IQ^{TM} SYBR Green PCR Master Mix (BIORAD) in a CFX96 Real-Time PCR Detection System (BIORAD) for the following genes using gene-specific primers (Table 1).

Table 1. Sequences of primers used in qRT-PCR

GENES	FORWARD PRIMER 5′-3′	REVERSE PRIMER 5′-3′
BOD1	GCTTACCAAAACCTGAGGCA	CCTGTCTACTCCAGCTTCCA
DIO2	CCCCAAGTTGCTTTGCTCAA	AGCAAGTCTACGCTGAGGAT
PTGS2	TCCCTTCCTTCGAAATGCAA	TCATCAGGCACAGGAGGAAG
SERPINB2	AACCCCAGGCAGTAGACTTC	TCCCCATCTACAGAACCTTCAG
FGF18	GGGCAAGGAGACGGAATTCT	AACCTTCTCGATGAACACACAC
WNT7A	CAATGGCCGCTGGAACTG	GCAATGATGGCGTAGGTGAA
KIF12	GCTTCTATGTGGAGCAGCTG	ACGGCTGATGTAAAGGGTGA
CDH6	ACAGTATTCTACAGGGACAGCC	CACTTGGTACTGCTCCCTGT
ZBED2	GACGAGGAAGAGGAGGGAAC	CTTGTTGTGGGGCATTGGAG
CHRD	CAGGAGTGGGGGACTAACC	CAGCACCTCAGCAAAGCCT
ANXA2	GTTCACGAAATCCTGTGCAA	TGGTCTTGATGGCTGTTTCA
PAX8	CCCTTCCAACACGCCACT	CTGCTTTATGGCGAAGGGTG
IPO8	AATTCCCAGTACGACAGGCAG	TCACGTATTTGCTGGCGATC
ITGB3	CTCATATAGCATTGGACGGAAGG	ACATTTTCAGTCACTGCAAAGAT
ITGAV	CGGATGTTTCTTCTCGTGGG	CCTCACAGATGCTCCAAACC
ABL	TGGAGATAACACTCTAAGCATAACTA	GATGTAGTTGCTTGGGACCCA

3.6 RNA-seq and data mining

After 24 hrs of transfection with siCTR and siPAX8 of FT194 and SKOV3 cells, 2 µg of total RNA was extracted and sent to the Genomics4Life Company (University of Salerno, Italy). Three independent silencing experiments were performed for each condition (siPAX8 or control) and processed using RNA-seq technology. The extracted RNA samples were sequenced by the Illumina HiSeq 1500 platform using a resolution at 100 base-pairs with paired-end reads. Analysis was performed using the RAP (RNA-Seq Analysis Pipeline) available on https://bioinformatics.cineca.it/ (D'Antonio et al. 2014). The sequences quality check, the mapping, the transcriptomes assembling, and the differential expression analyses were performed using the default

parameters on RAP. An alpha level of 0.05 was used for all the statistical tests. Gene expression data were submitted on Gene Expression Omnibus (GEO) database with accession number GSE79572.

3.7 Pathway analysis

Gene ontology (GO) and Panther pathway analysis have been performed using the GeneCodis tool (http:// genecodis.cnb.csic.es) previously described in references (Carmona-Saez et al. 2007; Tabas-Madrid et al. 2012).

3.8 Cytoscape analysis

The Cytoscape v3.6 Core, an open source software package (www.cytoscape.org/) with an accessible Java application programming interface (API) using the Java programming language has been used for the network analysis of our RNA-seq data. For the analysis the following parameters have been applied for investigating biological-molecular network between PAX8 and ITGB3:
Node: Represents single gene/protein
Color node: Represents only gene family and their molecular-biological pathways
Size node/ size label: Represents the gene expression level (up/down regulated) or the importance and strength of a node inside specific network
Edge: Represents the biological-molecular interactions
Color edge: Represents the importance and strength of molecular interactions
Size edge/ edge betwenness: Represents the weight of interactions
Length edge: Represents the position of node inside biological network
Directed network: Represents the direction of PPI between source-target
The molecular networks are realized applying several parameters to investigate new important biological interactions related to our gene/protein list used as starting point. Here, PAX8 has been used as the starting point to obtain the biological network.

3.9 Migration assay

Migration assays were performed using Ibidi cell migration technology (Ibidi, Martinsried, Germany). PAX8 was silenced in Primary hFTSECs, SKOV3, KURAMOCHI, OVSAHO and PEA1 cell lines as described before. After 24 hrs, both Scramble control and PAX8 silenced cells were seeded in each chamber at a density of 3×10^5 cells/reservoir in 70 μL of normal medium for 24 hrs. The medium was then replaced with fresh medium and the cells were treated with 10 ug/ml of Mitomicyn C (Sigma M4287-2MG) for 1 h at 37°C. After the incubation, the chambers were removed and cells were further incubated in normal medium. Cells were photographed (1:1 magnification), and the area covered by cells within a defined area in the gap measured using NIH ImageJ (rsb.info.nih.gov/ij) software.

3.10 Adhesion assay

Coverslips were coated with Fibronectin (10 μg/ml; Calbiochem, Cat N. 341635) or Collagen I (10 ug/ml; Invitrogen, Cat N. A10483-01) in PBS 1X for 1h at 37°C. PAX8 was silenced in Primary hFTSECs, SKOV3, KURAMOCHI, OVSAHO and PEA1 cell lines as described before. After 48 hrs, 40×10^3 of both scramble controls and PAX8 silenced cells were plated on the top of coated coverslips in triplicates for 2 hrs at 37°C. After incubation, the coverslips were washed with PBS 1X, fixed in 4% paraformaldehyde for 10 min and stained with HOECHST. The experiment was repeated three times (n = 3) for each cell lines. Images were acquired using Confocal microscope (ZEISS LSM 700). For each coverslip, 10 images were acquired and analyzed using ImageJ software.

3.11 Immunofluorescence and Confocal Laser Scanning Microscopy

After 24 hrs of transfection with siCTR and siPAX8 as described before, 50×10^3 of Primary hFTSECs cells and KURAMOCHI cells were spitted on glass coverslips and maintained in culture for 24 hrs at 37 °C.

The cells were fixed in 4% paraformaldehyde in PBS 1X for 20 min at room temperature and incubated for 30 min in 10% FBS in PBS 1X. The

coverslips were subsequently incubated for 1h with mouse monoclonal anti-$\alpha_v\beta_3$ LM609 (Millipore Co.) and rabbit polyclonal anti-PAX8 diluted, respectively, 1:100 and 1:1000 in 4% FBS in PBS 1X. After PBS washing, the cells were incubated for 30 min with Alexa Fluor-546 goat anti-mouse IgG (Vinci Biochem) Alexa Fluor-488 goat anti-rabbit IgG (Vinci Biochem) both diluted 1:200 in 4% FBS in PBS 1X. After final washings with PBS 1X, the coverslips were mounted on a microscope slide using a 50% solution of glycerol in PBS 1X with Heoechst (1:3000).

Experiments were carried out on an inverted and motorized microscope (Axio Observer Z.1) equipped with a 63X/1.4 Plan-Apochromat objective. The attached laser-scanning unit (LSM 700 4X pigtailed laser 405-488-555-639; Zeiss, Jena, Germany) enabled confocal imaging. For excitation, 405, 488 and 555nm lasers were used. Fluorescence emission was revealed by Main Dichroic Beam Splitter and Variable Secondary Dichroic Beam Splitter. Double and/or triple staining fluorescence images were acquired separately using ZEN 2012 software in the blue, green and/or red channels at a resolution of 1024 x 1024 pixels, with the confocal pinhole set to one Airy unit and then saved in TIFF format.

4. RESULTS

4.1 Identification of PAX8 downstream target genes in FT194 and SKOV3 cells by means of RNA-seq analysis

Recently, it has been suggested that high-grade serous ovarian carcinoma (HGSC) arises from precursor lesions in the Fallopian tube fimbria. PAX8 is a transcription factor expressed in Fallopian tube secretory cells and its expression is retained in HGSC cells confirming the tubal origin theory. The research group of Dr. Zannini has previously demonstrated that PAX8 has a pivotal role in the tumorigenic phenotype of ovarian cancer cells. Infact, PAX8 silencing in ovarian cancer cells strongly suppressed anchorage-independent growth *in vitro* and inhibited tumorigenesis *in vivo* in a nude mouse xenograft model (Di Palma et al. 2014). Based on these evidences, it is plausible that PAX8 is involved in the development and progression of ovarian cancer. However, its specific function in Fallopian tube secretory cells and ovarian cancer cells is still unknown.

To uncover the downstream gene network governed by PAX8 in both Fallopian tube secretory cells and ovarian cancer cells, we analyzed the transcriptome of Fallopian tube secretory cells (FT194) and ovarian cancer cells (SKOV3) using an RNA-seq strategy. To this end, FT194 and SKOV3 cells were transiently transfected with PAX8 siRNA (siPAX8) or siRNA Non Targeting (siCTR) as scramble. Three independent silencing experiments were performed for each cell line. After 24 hrs, total RNAs were prepared and sent to the Genomix4life Company (University of Salerno) and submitted to the Illumina HiSeq 1500 platform for sequencing libraries generation. The raw data were analysed in collaboration with Prof. Cocozza (Department of Molecular Medicine and Medical Biotechnology, University of Naples Federico II, Naples, Italy) and processed with FDR-adjusted p-value ≤ 0.05. The results showed that 467 genes were significantly modulated by PAX8 silencing in both cell lines. The decrease of PAX8 expression was analyzed for confirmation of the silencing and 80% of decrease in siPAX8-cells with respect to siCTR-cells demonstrated efficient silencing.

In FT194 cells 166 genes were affected by PAX8 silencing of which 119 were downregulated and 47 were upregulated with respect to the scramble. In SKOV3 cells, 301 genes were significantly modulated after PAX8 silencing and among the total genes, 214 genes were

downregulated and 87 were upregulated with respect to the scramble (Fig. 5).

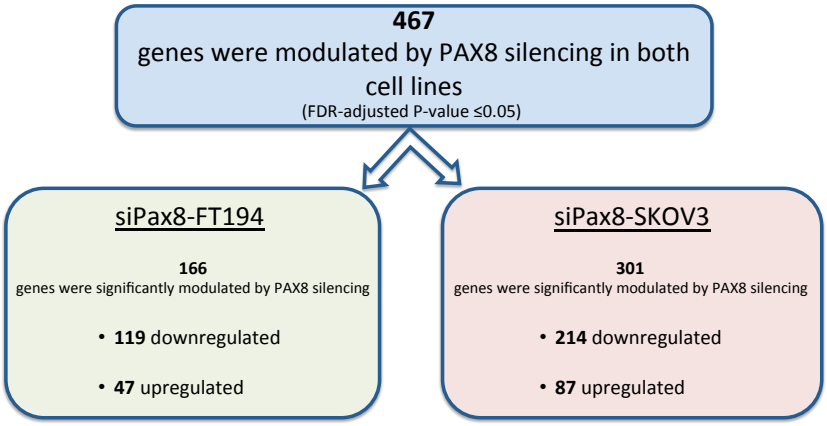


Fig. 5 Schematic representation of the genes significantly modulated after the RNA-seq analysis. The genes were divided in downregulated and upregulated in both siPAX8-FT194 and siPAX8-SKOV3 cells.

4.2 Identification of shared and exclusive sets of genes in FT194 and SKOV3 cells regulated by PAX8

To understand how PAX8 is involved in both physiological and pathological states, Venn diagrams were constructed to highlight the common and distinct sets of genes affected by PAX8 silencing in FT194 and SKOV3 cell lines (Fig. 6). Among the significantly downregulated genes, 167 were exclusively downregulated in siPAX8-FT194, 72 genes in siPAX8-SKOV3 cells and 47 were commonly downregulated in both (Fig 6A). With respect to the significantly upregulated genes, 15 were commonly upregulated in both cell lines and 72 and 32 genes were exclusively upregulated in siPAX8-FT194 and siPAX8-SKOV3 cells respectively (Fig 6B). The complete lists of shared and distinct genes modulated upon PAX8 silencing in both these cell lines are reported in Tables 2A and 2B.
In particular, the common down- and up-regulated genes, such as PSAP, FGF18, CDH6, ROR1, RBPJ and DNMT3B, could be looked as promising putative targets of PAX8 with most of them already reported having roles in cell proliferation, cell survival and tumorigenic process. At the same time, the genes modulated upon PAX8 silencing exclusively

expressed in FT194 or in SKOV3 cells could reflect the continuous process that distinguishes itself from the precancerous to the cancerous condition. In addition, there is a possibility that the tumorigenic process might itself promote the expression of some genes making them available for PAX8 transcriptional regulation.

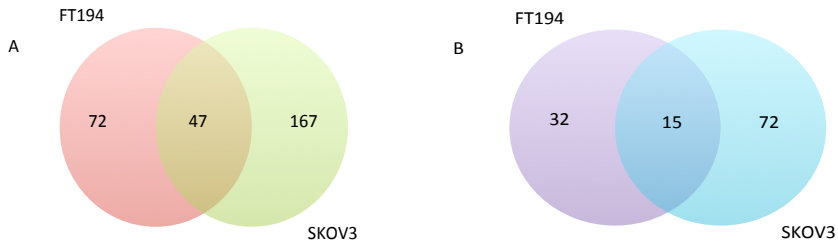


Fig.6 Venn diagram of modulated genes upon PAX8 silencing in FT194 and SKOV3 cells:
(A) Overlap and differences of downregulated genes following PAX8 silencing between FT194 and SKOV3 cells
(B) Overlap and differences of upregulated genes following PAX8 silencing between FT194 and SKOV3 cells.

Table 2A: List of shared and exclusive genes downregulated upon PAX8 silencing in FT194 and SKOV3 cells.

NAME	TOTAL	DOWNREGULATED GENES
SKOV3-FT194	47	NUP35 ANXA2 HDGFRP3 STX3 DUSP11 AP1G1 ENPP4 PHLDA3 PSAP ENPP1 EPRS RHPN2 C10orf46 GPR63 CDK2AP1 H2AFY2 PPME1 LMLN KITLG CDH6a FAM107B C1orf186 BLCAP TEX30 LONRF1 DIAPH1 FGF18 TCN2 ROR1 AKR1B1 TGOLN2 KAZN PHTF2 ARL4C MAL ADCY9 PPAP2B PAX8 ARL5A MAL2 MRE11A HTATSF1 GPR56 PTGIS ANXA2P2 ABCC4 SLC35B4
SKOV3	167	ACTB TSPAN1 RAB12 OSBPL11 L1CAM OSBPL10 LRRC20 RRAD ANKRD34B RNF38 HES2 SMC5 FAM3C PRKAA2 KCNH3 LMAN1 ST3GAL2 AFF1 TAF4B IFNGR1 FHDC1 CGNL1 CA13 SYPL1 MKNK2 SLC39A10 AMMECR1 ZNF114 GAS2L3 CERK BAG4 SASH1 C6orf228 TBC1D13 C7orf29,LRRC61 TUBB2B GTF2E1 ABCC3 PLCH1 CLDND1 MBP CYB5R4 SLC39A14 AKR1B10 CELSR2 PDZK1 FOXN3 PIK3R1 GRIN2B CIAPIN1 CORO1C RNFT1 UPP1 SHROOM2 FAM45A,FAM45B ANKRD33B ZNF702P ID4 PPM1B PER1 SLC44A1 CDH16 PCDH20 ADAM10 RNF130 CBS KIF12 KLHL15 ZNF28 ATP8B2 EGFR MIR4723,TMEM199 DICER1 DPP4 ITGB3 DENND1B SGK2 BASP1P1 USP18 RAB3D DCUN1D4 SNTB1 PGGT1B CNKSR3 CHST15 MCAM TYW1 GCH1 TRIM2 C12orf23 FNDC5 IGFN1 USP2 TERF1 SORT1 CRYAB GMCL1 WNT7A MXRA5 AGL RNF144B ASRGL1 MPHOSPH6 QPRT AATF LIN7C NXT2 C2orf72 RRM2 BCAT1 SLC7A11 ADAMTS9 TMSB4X TPK1 KPNA6 ATP8A1 CHST2 THBS1 CYP4F11 RNF145 ADAMTS5 FLJ26245 SYTL2 UBE2H DCAF12L1 RAB11FIP2 ABI2 PTPRB FIGN PAFAH1B2 TCF12 PMAIP1 WDR44 DCUN1D3 SLC30A6 EPHA4 NEBL DSC2 CNOT6 ZNF611 MYO10 MAP2 KCNS3 SLC26A2 HDHD2 SLAIN1 PLXNC1 MGST1 NUPL1 COL12A1 KCTD5 RCBTB2 CANT1 MTPN FN1 UBE2D1 ZCCHC24 TMTC2 TRIM24 FAM174B C10orf26 TMCO7 GUCY1B3 ARHGEF1 ANKRD52 RC3H2 ZNF618
FT194	72	KLHL13 ZNF185 PPARGC1A WASF1 PLAU KLHL14 TLL2 CHRD CLGN NGFR ARHGEF37 FLG GAS7 TGFBR3 RGS20 ILDR2 SPON1 MMD CD24 AP1M2 TNFSF4 THY1 ADAMTS14 ZBED2a PLCB4 ADAMTS1 LOC643201 NOV MPP7 SHISA2 ST3GAL1 SOX17 GDF6 ANK3 RASGEF1B CNTN4 C13orf15 SULF2 ADORA1 MEGF9 KLRG2 ITGB8 DANCR,SNORA26 SLC6A6 BAALC CTHRC1 G0S2 TMEM117 AIF1L GJB2 PRKAG2 FAT2 MST1 DCDC2 OXTR SDC2 INHBB KIAA1456 BTBD11 CA2 NID2 SLC47A1 CDH5 PDE1A COL13A1 IGFBP5 TMEM170B CTGF EPHB1 GPRC5C DKK1 CDKN1C

Table 2B: List of shared and exclusive genes upregulated upon PAX8 silencing in FT194 and SKOV3 cells.

NAME	TOTAL	UPREGULATED GENES
SKOV3-FT194	15	ANKRD1 F3 G3BP2 TGFB2 MAPK1IP1L TOMM20 MBTPS1 DNMT3B PTPN1 MSRB3 MET RBPJ TCEB3 WDR1 STX12
SKOV3	72	DIO2 ZCCHC3 NTN4 NGRN TRAPPC2 CALU MOK DCBLD1 PTPMT1 FGF1 C1D AHRR,PDCD6 LOC100507412,RN45S PCSK7 CPA4 CIRBP SERPINE1 C6orf120 PODXL CCDC80 DOCK10 PYGO2 P4HA3 MCFD2 DSEL MYPN LPXN CCND3 CHFR MAP2K4 GRWD1 CCBE1 UBE2G1,ZZEF1 BOD1 NCF2 ODZ2 ERRFI1 CCIN SERPINB5 ATG12 CGB8 FLRT2 AIM1 ROS1 C7orf58 ALS2CL FRMD6 KRT5 SSFA2 PIP4K2A SOCS7 NT5E ESYT2 SMAP1 ARTN SCRN1 DKK1 EFEMP1 RFK SGK1 CCDC68 CRK FOXD1 PGM2L1 PCDH10 SEMA7A ZFAND3 IFFO2 CCNC RCAN1 KHNYN POLR3F
FT194	32	CMPK2 RAD54L2 DDX58 IFIT1 SP110 PTPN13 DCN PAPPA RHOB CCL20 GBP1 SERPINB2 OAS3 PDZD2 IFIH1 NCOA7 OAS2 RSAD2 PTGS2 MX1 ZCCHC2 CYP1B1 DHX58 DDX60L HMGA2 FARP2 MX2 NRK FPR1 THBD ST6GALNAC5 THBS2

4.3 Validation of RNA-seq data by qRT-PCR

To validate the data obtained by the RNA-seq analysis, we performed qRT-PCR for 13 representative genes, including PAX8. For all the selected genes, we firstly confirmed the differential expression between SKOV3 cells and FT194 cells. The results show that PAX8 is expressed at similar levels in these two cell types, supporting the hypothesis that PAX8 is not overexpressed in epithelial ovarian carcinoma but its expression is retained from the Fallopian tubes epithelial cells, the suggested cell of origin (Fig. 7). Firstly, according to the RNA-seq results, the genes analyzed by qRT-PCR follow the same trends in FT194 and SKOV3 cells confirming their differential expression. Secondly, the same genes were validated in siPAX8-FT194 and siPAX8-SKOV3 cells with respect to the siCTR-cells 24 hrs, 48 hrs and 72 hrs after silencing (Fig 8A and 8B). In accordance with the RNA-seq results, we confirmed that genes like PAPPA, CHRD, ZBED2 and SERPINB2 were preferentially or exclusively expressed in FT194 cells and after PAX8 silencing CHRD and ZBED2 were downregulated and PAPPA and SERPINB2 were upregulated with respect to the siCTR-FT194 cells. In parallel, genes like BOD1, DIO2, WNT7a and KIF12 are preferentially or exclusively expressed in SKOV3 cells. Upon PAX8 silencing, BOD1 and DIO2 were upregulated and WNT7a and KIF12 were downregulated in siPAX8-SKOV3 cells with respect to siCTR-SKOV3 cells. The genes commonly expressed in both these cell lines were ANXA2, ROR1, CDH6, and FGF18 and all were downregulated after the PAX8 silencing. To strengthen our observations, we successfully validated the differential expression of all the above mentioned genes in human Fallopian tube RNA and OVCAR-3, PEA1, PEO14 ovarian cancer cell lines (Fig. 9) and for PEA1 24 hrs after transient transfection with PAX8 siRNA or scramble siRNA (Fig. 10).

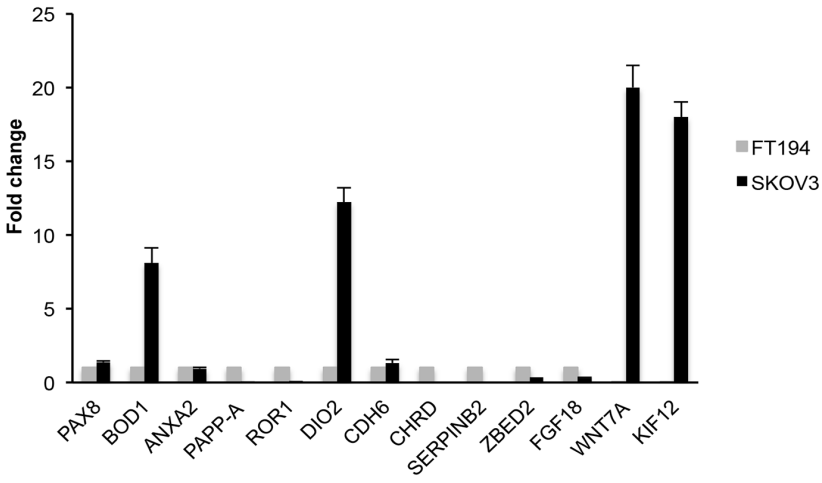
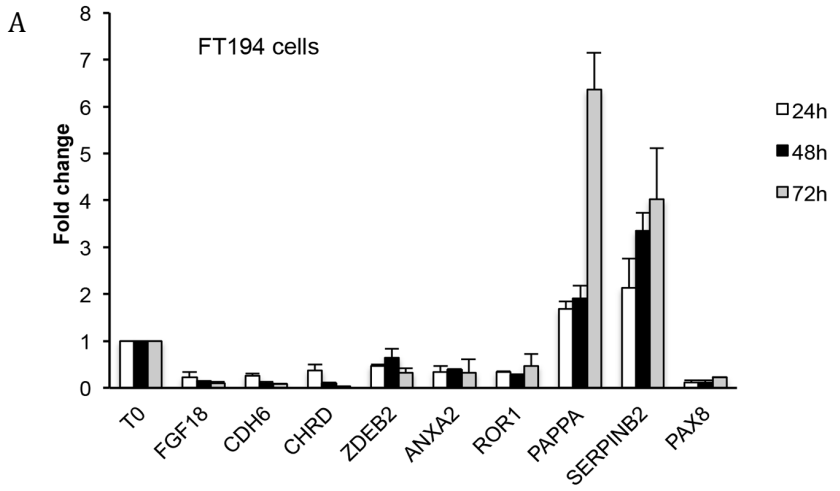


Fig. 7 Expression level of 13 genes measured on total RNA prepared from FT194 and SKOV3 cells. The values are means ±SD of three experiments in duplicate, normalized by the expression of IPO8 and expressed as fold change with respect to FT194 cells.



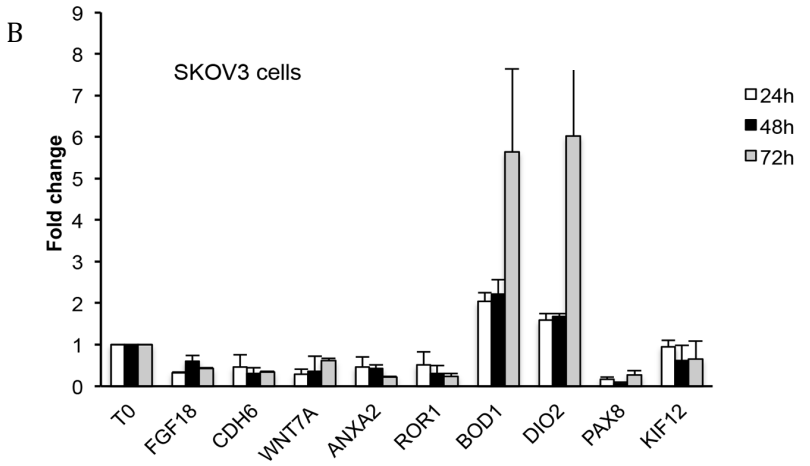


Fig. 8A and 8B Expression levels of some representative genes measured on total RNA prepared from FT194 and SKOV3 cells transiently transfected with PAX8 siRNA or scramble siRNA 24 hrs (white bars), 48 hrs (black bars) and 72 hrs (grey bars) after transfection. The values are means ± SD of three independent experiments in duplicate, normalized by the expression of IP08 and expressed as fold change with respect to the cells transfected with the scramble siRNA, whose value was set at 1.0. *p*-value was calculated by *t*-test $0.001 \leq p \leq 0.1$.

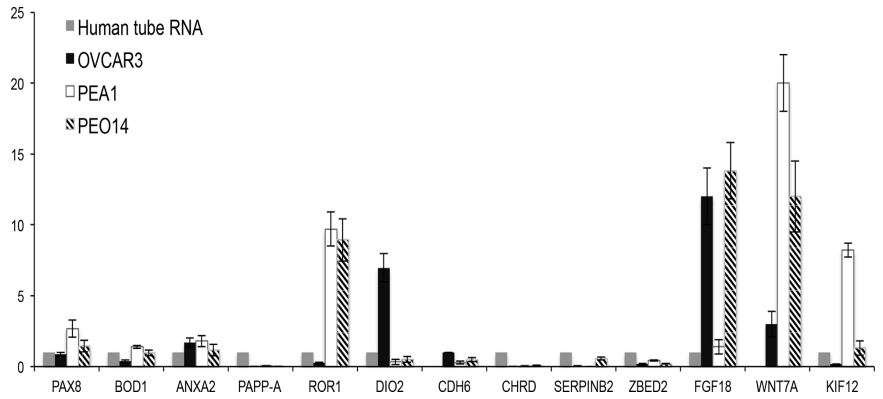


Fig. 9 Expression levels of 13 genes measured on total RNA prepared from human Fallopian tubes, OVCAR3, PEA1 and PE014 cells. The values are means ± SD of three independent experiments in duplicate, normalized by the expression of IP08 and expressed as fold change with respect to human Fallopian tubes, whose value was set at 1.0. *p*-value was calculated by *t*-test $0.001 \leq p \leq 0.1$.

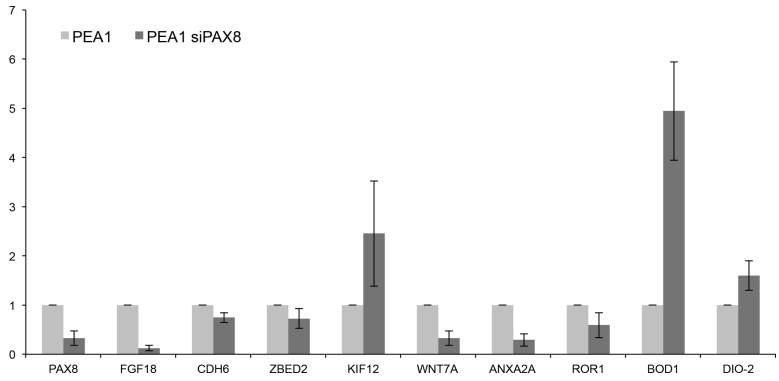


Fig. 10 Expression levels of some representative genes measured on total RNA prepared from PEA1 cells 24 hrs after transient transfection with PAX8 siRNA or scramble siRNA. The values are means ± SD of three independent experiments in duplicate, normalized by the expression of IP08 and expressed as fold change with respect to the cells transfected with the scramble siRNA, whose value was set at 1.0. *p*-value was calculated by *t*-test $0.001 \leq p \leq 0.1$.

4.4 Pathways and biological processes modified by PAX8

The pathways regulated by PAX8 in ovarian cancer cells and Fallopian tube secretory cells are still unexplained. To categorize PAX8 associated pathways in siPAX8-FT194 and siPAX8-SKOV3 cells, Gene Ontology (GO) and Panther pathway analyses were performed using Gene annotations co-occurrence discovery web-based tool (GeneCodis; http://genecodis.dacya.ucm.es/). Processing all the dysregulated genes (301 for SKOV3 and 166 for FT194), several significant GO categories were enriched. In details, signal transduction, cell adhesion, blood coagulation, and multicellular organismal development were statistically enriched in both cell lines. To underline the most important biological pathways involved in siPAX8-FT194 and siPAX8-SKOV3 cells, 10 statistically enriched categories with highest number of genes and lowest corrected p-value were chosen (p-values have been obtained through Hypergeometric analysis corrected by FDR method) (Fig. 11A and 12A). In Table 3 and 4 are reported the number of the genes, the corrected p-values and the complete list of the genes for the 10 biological process enriched in FT194 and SKOV3 cells after PAX8 silencing. It is important to note that in the SKOV3 cancer cell line, the silencing of PAX8 affected the migration bioprocess. The migrating ability of cancer cells is an important feature in tumor progression and the consequent metastasis of

the disease. Particularly, the GO results showed that both “cell migration” and the “positive regulation of cell migration” categories displayed 5% of the transcriptome changes in SKOV3 cells upon PAX8 silencing. Interestingly, the “cell adhesion” GO category affected both siPAX8-FT194 and siPAX8-SKOV3 cells and displayed 16% of overall transcriptome changes in SKOV3 cells and 14% in FT194 cells following PAX8 silencing. Panther pathway analysis was performed to further investigate the functional associations of the modulated genes following PAX8 knockdown. The results showed that the highest differential transcriptome changes in both cell lines were found in Wnt, Cadherin, Integrin, TGF-beta signaling pathways and angiogenesis process (Fig 11B and 12B). The results showed in GO and Panther analyses are consistent with each other, probably due to the strong downregulation of genes like Wnt7a, Cdh6, Cdh16, βActin and Integrin β_3. The modulation of these processes by PAX8 could highlight a new role of this transcription factor in HGSC development.

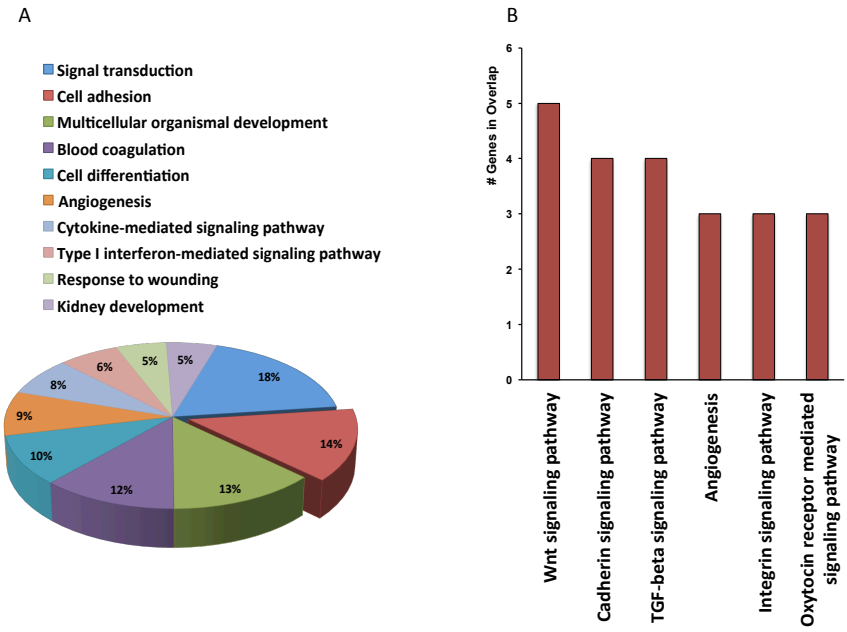


Fig. 11 Biological processes and pathways altered in siPAX8-FT194 cells. (A) GO categories enriched for genes modulated upon PAX8 silencing in FT194 cells. Pie-chart with the 10 GO enriched categories with highest number of genes and lowest FDR corrected p-value (<0,05). (B) Panther pathways enriched for genes modulated upon PAX8 silencing in FT194 cells.

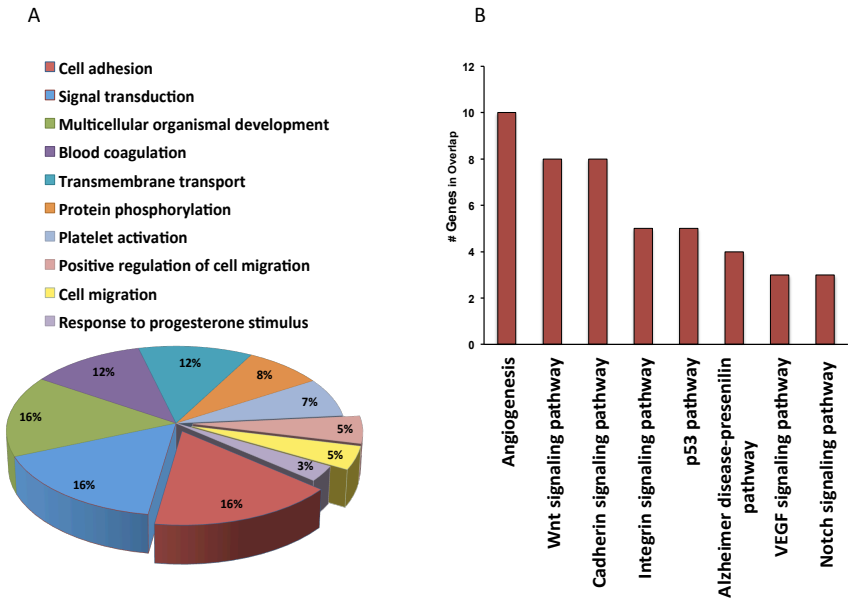


Fig. 12 Biological processes and pathways altered in siPAX8-SKOV3 cells. (A) GO categories enriched for genes modulated upon PAX8 silencing in SKOV3 cells. Pie-chart with the 10 GO enriched categories with highest number of genes and lowest FDR corrected p-value (<0,05). (B) Panther pathways enriched for genes modulated upon PAX8 silencing in SKOV3 cells.

Number of genes	Corrected p-values	GO category	List of genes
17	0.00316167	Signal transduction	CCL20, SP110, ADORA1, KITLG, DKK1, NGFR, EPHB1, RHPN2, IGFBP5, ADCY9, PDE1A, TNFSF4, FGF18, PLAU, MET, ANK3, MX1
13	0.000644665	Cell adhesion	KITLG, CDH6, CDH5, ITGB8, GPR56, CNTN4, NID2, RHOB, LMLN, PDZD2, THBS2, FAT2, CTGF
12	0.0325062	Multicellular organismal development	HMGA2, DKK1, NGFR, GAS7, TLL2, ITGB8, RHOB, SHISA2, PAX8, MET, FLG, COL13A1
11	0.00228239	Blood coagulation	SERPINB2, PSAP, PTPN1, RHOB, ABCC4, PDE1A, PLAU, TGFB2, WDR1, F3, THBD
9	0.022979	Cell differentiation	NGFR, TLL2, ILDR2, MAL, RHOB, PAX8, PAPPA, INHBB, COL13A1
8	0.000775251	Angiogenesis	SOX17, EPHB1, ANXA2, CYP1B1, THY1, FGF18, PLAU, TGFB2
7	0.00289927	Cytokine-mediated signaling pathway	MX2, OAS3, PTPN1, OAS2, IFIT1, MX1, GBP1
6	0.000613639	Type I interferon-mediated signaling pathway	MX2, OAS3, PTPN1, OAS2, IFIT1, MX1
5	0.00232654	Response to wounding	NGFR, TGFB2, MET, F3, CTGF
5	0.00277451	Kidney development	SULF2, PAX8, DCN, CA2, ADAMTS1

Table 3: 10 statistically enriched GO categories with highest number of genes and lowest corrected p-value were chosen (p-values have been obtained through Hypergeometric analysis corrected by FDR method). In the list are reported the number of the genes, the corrected p-values and the complete list of the genes enriched in FT194 cells after PAX8 silencing.

Number of genes	Corrected p-values	GO category	List of genes
22	1.13382e-05	Cell adhesion	L1CAM, MCAM, THBS1, DCBLD1, KITLG, DSC2, LPXN, CDH6, PCDH20, DPP4, ITGB3, FLRT2, CDH16, PLXNC1, GPR56, FN1, CELSR2, AATF, EPHA4, LMLN, PCDH10, COL12A1
22	0.0299607	Signal transduction	MAP2K4, IFNGR1, KITLG, LPXN, DKK1, MYO10, ARTN, RCAN1, FGF1, PRKAA2, PIK3R1, RHPN2, CORO1C, ODZ2, CCND3, PLXNC1, ADCY9, FGF18, EGFR, CANT1, MET, RRAD
21	0.00852199	Multicellular organismal development	TCF12, L1CAM, SHROOM2, SORT1, WNT7A, DKK1, NGRN, FGF1, GMCL1, CCBE1, PLXNC1, FAM3C, CELSR2, CHST2, EPHA4, PAX8, CCIN, EGFR, MET, ADAMTS9, SEMA7A
16	0.000592788	Blood coagulation	LMAN1, L1CAM, THBS1, SLC7A11, PIK3R1, CRK, GUCY1B3, PSAP, PTPN1, FN1, CALU, ABCC4, TGFB2, WDR1, F3, SERPINE1
16	0.0113474	Transmembrane transport	KCNS3, SLC44A1, SLC26A2, SLC7A11, KCNH3, ADCY9, ATP8A1, ATP8B2, SLC35B4, ABCC4, SLC39A14, SLC30A6, ABCC3, SLC39A10, NUPL1, NUP35
11	0.0303045	Protein phosphorylation	SGK1, MAP2K4, ADAM10, CDK2AP1, MKNK2, PRKAA2, CCND3, ROS1, EGFR, TGFB2, SGK2
10	0.00612199	Platelet activation	THBS1, PIK3R1, GUCY1B3, PSAP, FN1, CALU, ABCC4, TGFB2, WDR1, SERPINE1
7	0.00757332	Positive regulation of cell migration	ADAM10, THBS1, FGF1, PIK3R1, PODXL, EGFR, F3
6	0.0220931	Cell migration	SHROOM2, THBS1, PODXL, FN1, TGFB2, ABI2
4	0.010583	Response to progesterone stimulus	THBS1, NCF2, PIK3R1, TGFB2

Table 4. 10 statistically enriched GO categories with highest number of genes and lowest corrected p-value were chosen (p-values have been obtained through Hypergeometric analysis corrected by FDR method). In the list are reported the number of the genes, the corrected p-values and the complete list of the genes enriched in SKOV3 cells after PAX8 silencing.

4.5 Analysis of the migration ability of Fallopian tube secretory cells and ovarian cancer cell lines after PAX8 silencing

One of the most important feature of ovarian cancer is its high rate of metastasis. In fact, when this disease is diagnosed, the patient already has ovarian mets in the peritoneum and in other organs. This happens mainly due to the total lack of symptoms and absence of early stage biomarkers, resulting in very late diagnosis.
Therefore, cellular migration and adhesion are important characteristics in the HGSC progression and metastasis and it is important to consider them as closely related. Cell migration requires a dynamic interaction between the cells and the substratum on which they attach and move. Changes in the adhesion molecule repertoire may correspond to changes in migratory properties conferring a more invasive phenotype to cancer cells. For further experiments, primary human fallopian tube secretory cells (primary hFTSCs) that represent the normal physiology has been used. To explore the migratory ability of Fallopian tube secretory cells and epithelial ovarian cancer cells, before and after PAX8 silencing, migration assays were performed using the Ibidi cell migration technology. The results showed that PAX8 silencing significantly affects the migratory properties showing a reduction in the cell migrated area with respect to siCTR cells in Primary hFTSEC and all epithelial ovarian cancer cells (SKOV3, KURAMOCHI, OVSAHO and PEA1) (Fig 13).

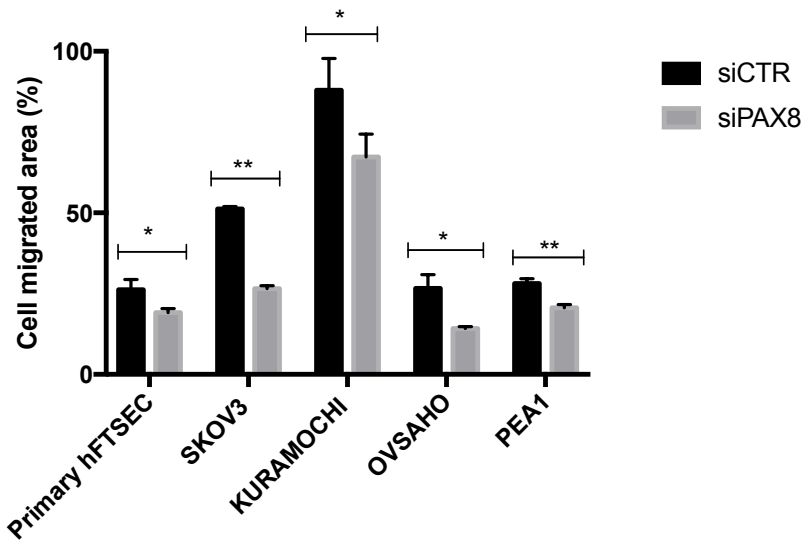
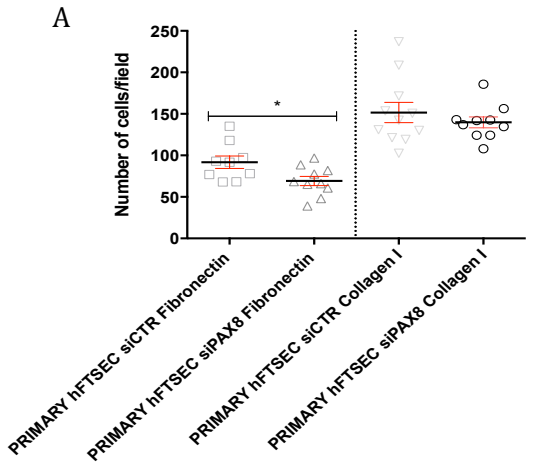


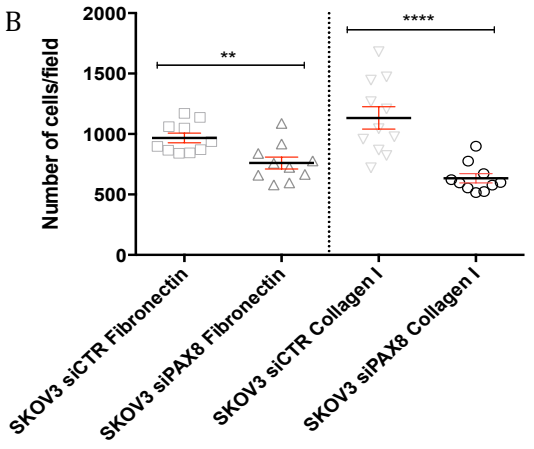
Fig. 13 Effect of PAX8 silencing on the migration ability of Primary hFTSEC, SKOV3, KURAMOCHI, OVSAHO and PEA1 cell lines. The cell migrated area was measured after 48 hrs of transient transfection with PAX8 siRNA (grey bars) or scramble siRNA (black bars). The values are means ± SD of three independent experiments normalized with respect to the cells transfected with the scramble siRNA. *p*-value was calculated by *t*-test $p \leq 0.1$.

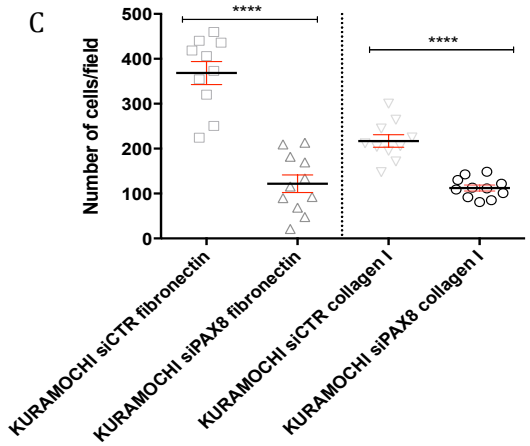
4.6 Analysis of the cell adhesion ability of the Fallopian tube secretory cells and ovarian cancer cell lines after PAX8 silencing

The RNA-seq analysis showed that PAX8 is able to modify the expression of several adhesion molecules like Wnt7a, Cdh6, Cdh16, βActin and Integrin β_3 genes. Whether this functionally affects the adhesive properties of ovarian tumor cells is not clearly understood. Therefore, to understand the consequences of the loss of PAX8 in cell-matrix adhesive properties, the ability of the cells to adhere to different extracellular matrices (ECM) was addressed. The adhesive abilities of the Primary hFTSEC, SKOV3, KURAMOCHI, OVSAHO and PEA1 cell lines were evaluated through cell adhesion assays using Fibronectin and Collagen I as substrates to mimics the ECM. Upon PAX8 silencing the Primary hFTSEC, SKOV3, KURAMOCHI, OVSAHO and PEA1 cell lines adhered more slowly when cultured on Fibronectin and Collagene I-coated coverslips with respect to siCTR-cells and showed significantly reduced adhesion after 2 hrs (Fig. 14 A, B, C, D, E). Overall, these results

suggest that PAX8 silencing could modify the expression of certain adhesion molecules in epithelial ovarian cancer cell lines like Integrins. Interestingly, Integrin expression is directly implicated in the progression of tumor and in formation of metastases.



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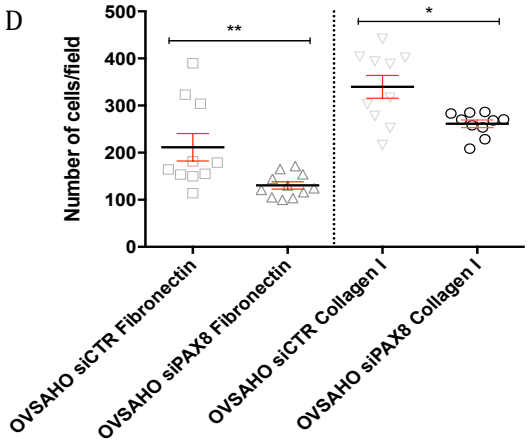
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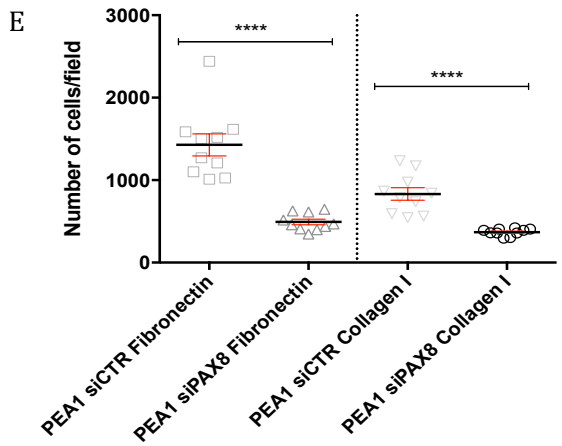

Fig. 14 Effect of PAX8 silencing on the adhesion ability to Fibronectin or Collagen I of Primary hFTSEC (A), SKOV3 (B), KURAMOCHI (C), OVSAHO (D) and PEA1 (E). The values are means ± SD of three independent experiments normalized with respect to the cells transfected with the scramble siRNA. *p*-value was calculated by *t*-test $p \leq 0.1$.

4.7 PAX8 modulates tumor cell adhesion by regulating Integrin β3 expression

The altered interaction upon PAX8 silencing of Primary hFTSECs and ovarian cancer cells with ECM suggest a possible effect on the Integrin function. The RNA-seq analysis shows that Integrin β_3 is amongst the genes significantly downregulated after PAX8 silencing in SKOV3 cells. Real-time qPCR was performed on Primary hFTSEC, SKOV3, KURAMOCHI, OVSAHO and PEA1 cell lines to understand if the expression of Integrin β_3 (ITGB3) was modified after PAX8 silencing. All the cell lines were transfected with siCTR and siPAX8 as previously described. After 48 hrs of transfection, cells were harvested, the RNA extracted and cDNA synthetized. The results of the Real-time qPCR analysis confirmed the reduction in the expression levels of PAX8 in all siPAX8 cells when compared to siCTR cells. Then, the expression level of ITGB3 was examined and interestingly, ITGB3 expression level showed strong decrease in all cell lines after PAX8 silencing (Fig. 15).

ITGB3 is reported to bind to only two other Integrins: Integrin α_{IIb} and integrin α_v. The expression of $\alpha_{IIb}\beta_3$ dimers is restricted to cells of the

megakaryocyte lineage and is required for platelet aggregation (Giancotti and Ruoslahti 1999). The $\alpha_v\beta_3$ integrin is expressed on proliferating endothelial cells and some cancer cells. There is evidence for the role of $\alpha_v\beta_3$ in multiple mechanisms of tumor growth and invasion, including interaction with ECM components, matrix metalloproteinase 2, platelet-derived growth factor, insulin, VEGF receptors, and prevention of apoptosis (Kris A DeMali et al. 2003). Recently, its expression is observed in ovarian cancer cells and its potential as a therapeutic target for blocking tumor-induced angiogenesis is being considered (Kobayashi et al. 2017). To this end, a Real-time qPCR was performed to analyze the expression level of Integrin α_v (ITGAV) after PAX8 silencing. No change in the expression level of ITGAV was found after PAX8 silencing in all the cell lines (Fig. 15).

To investigate the modulation of the Integrin $\alpha_v\beta_3$ heterodimer by PAX8, an immunofluorescence checking Integrin $\alpha_v\beta_3$ expression was performed on Primary hFTSECs and KURAMOCHI before and after PAX8 silencing. After 48 hrs of transfection with siPAX8 and siCTR, the cells were fixed on coverslips as described before and were stained with Anti-PAX8, Anti-Integrin $\alpha_v\beta_3$ antibody and HEOCHST for the nuclei. As showed in Figure 14, the lower intensity of the signal of PAX8 antibody in siPAX8-Primary hFTSECs and siPAX8-KURAMOCHI cells was a confirmation of PAX8 silencing (Fig. 16). Interestingly, the same result was observed for the signal of the Integrin $\alpha_v\beta_3$ antibody in both silenced cell types with respect to the control cells. In conclusion, we have established a correlation between PAX8 and Integrin $\alpha_v\beta_3$. This should be further explored to get better insights about the role of PAX8 in HGSC development and metastasis.

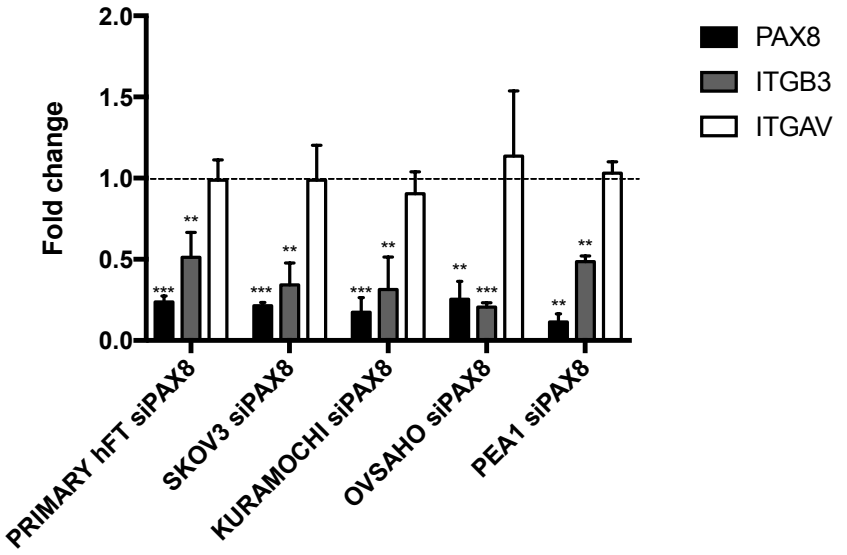

Fig. 15 Expression levels of PAX8 (black bars) ITGB3 (gray bars) and ITGAV (white bars) genes measured on total RNA prepared from Primary hFTSEC, SKOV3, KURAMOCHI, OVSAHO and PEA1 cells after 48 hrs of transiently transfection with PAX8 siRNA (siPAX8) or scramble siRNA (siCTR). The values are means ± SD of three independent experiments in duplicate, normalized by the expression of ABL and expressed as fold change with respect to the siCTR cells, whose value was set at 1.0. *p*-value was calculated by *t*-test $0.001 \leq p \leq 0.1$.

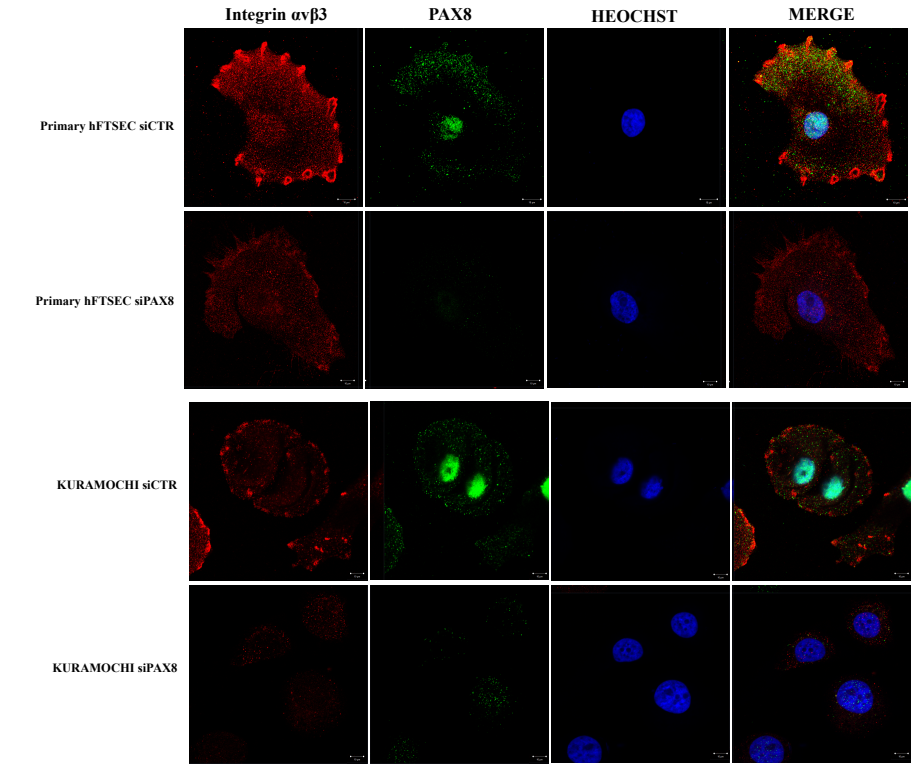

Fig. 16 Primary hFTSEC and KURAMOCHI cells were transfected with scramble siRNA (siCTR) and PAX8 siRNA (siPAX8) and after 24 hrs were plated on coverslips and maintained in culture for 24 hrs. The confocal fluorescence analysis was performed for Integrin $\alpha_v\beta_3$ (red channel) and PAX8 (green channel). HEOCHST (blue channel) was used to locate the nuclei (scale bar 10 μm). The images are representative of three independent experiments.

4.8 PAX8-ITGB3 molecular networks by Cytoscape

To further investigate the PAX8-ITGB3 network that possibly governs migration and adhesion in the Ovarian carcinoma context, we reanalyzed our RNA-seq data using the Cytoscape platform. To begin understanding the possible interactors of PAX8, we used Cytoscape to perform network analysis before and after PAX8 silencing in SKOV3. As a validation of our experimental results, ITGB3 appeared as one of the most probable first interactors of PAX8 in SKOV3 (Figure 17). To have better insights into the PAX8-ITGB3 regulatory network, we performed a putative gene network analysis involving ITGB3 as a mediator of PAX8 in cell-cell

interaction (contact inhibition) and cellular adhesion (tumor growth). Figure 18, shows the indirect targets of PAX8 regulated by its first interactor, ITGB3. The targets of ITGB3 show two kinds of interactions – radially pointed outwards and circularly interlinked – demonstrating different regulatory pathways. Most of these targets through ITGB3 are involved in tumor metastasis by various mechanisms like angiogenesis, cell migration, contact inhibition. It is important to note that the thickness of the arrow indicates the strength of the interaction and the color of arrows indicates the biological importance of the interactions. It is further interesting to note that upon PAX8 silencing in SKOV3 all its first interactors including ITGB3 and its associated targets are muted (Fig. 19). This is demonstrated by the decrease in cell adhesion and migration upon PAX8 silencing, which is reflected again on the network analysis of PAX8 –ITGB3 where the network collapses and other interaction are formed because there is no PAX8 or ITGB3 to regulate the network.

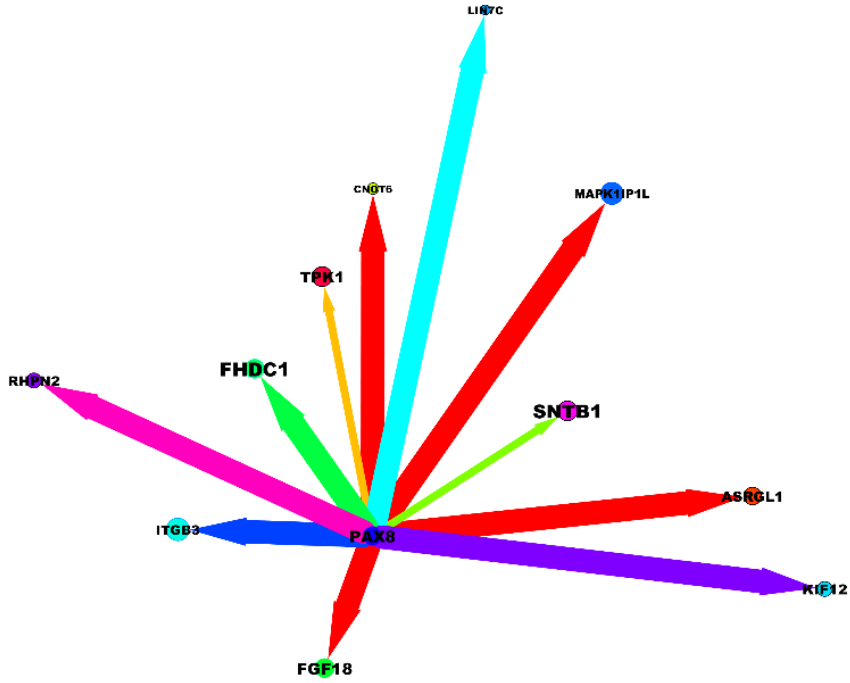

Fig. 17 Cytoscape analysis of PAX8-ITGB3 network in SKOV3 cells.

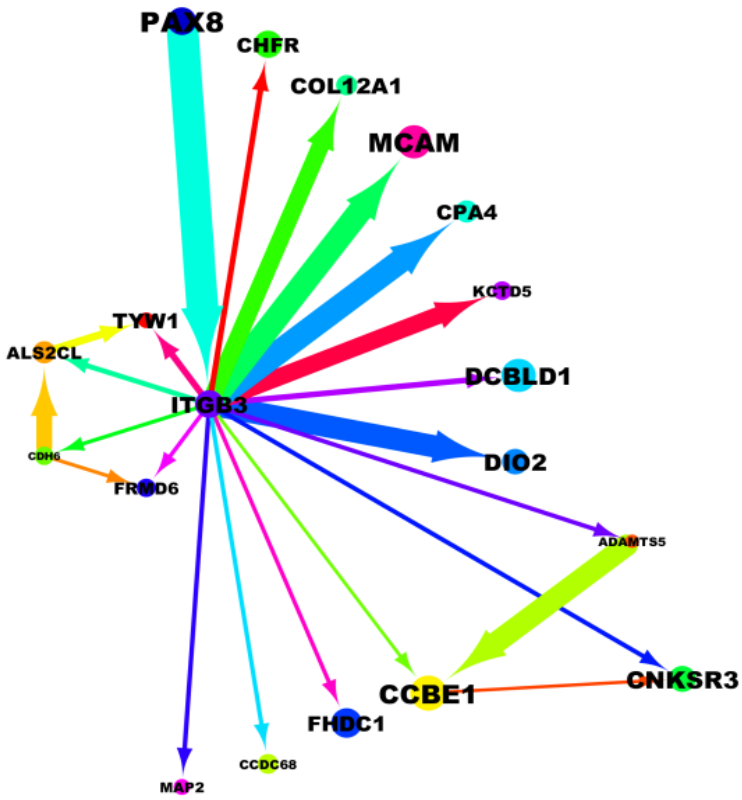

Fig.18 Cytoscape analysis of PAX8-ITGB3 network before PAX8 silencing in SKOV3 cells.

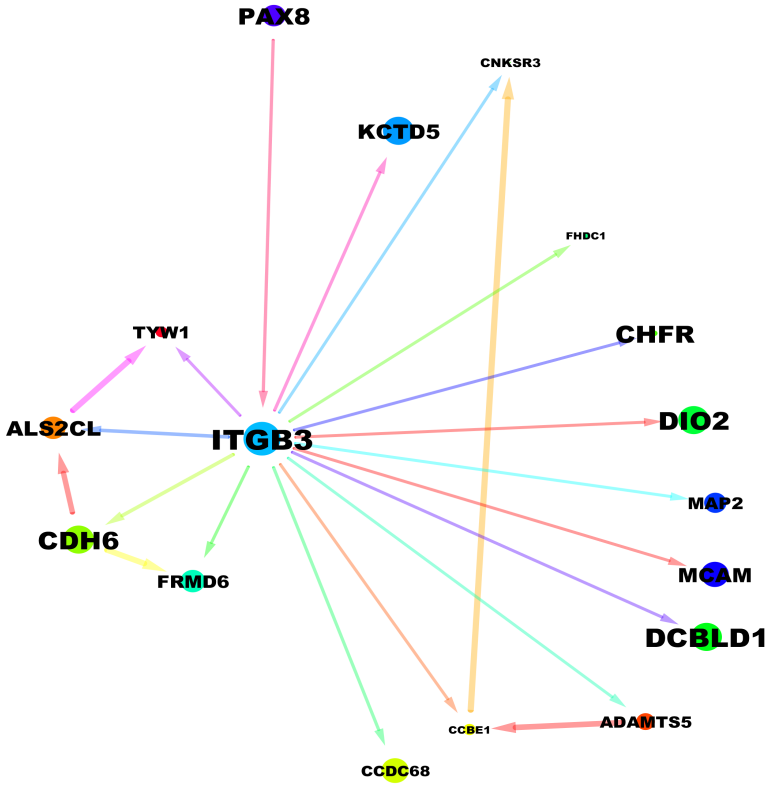

Fig.19 Cytoscape analysis of PAX8-ITGB3 network after PAX8 silencing in SKOV3 cells.

5. DISCUSSION AND CONCLUSION

Epithelial ovarian cancer (EOC) is the one of the most common gynecological malignancies and the fifth leading cause of cancer-related death in women (Siegel et al. 2015). EOC is a highly metastatic cancer characterized by widespread peritoneal dissemination and ascites accumulation. It is typically diagnosed in advanced stages due to earlier symptoms (McKnight et al. 2010). Currently, there are no early stage biomarkers available and the standard treatment of EOC is surgery followed by repeated cycles of platinum- and taxane-based chemotherapy. However, the 5-year survival rate of patients with advanced-stage EOC is only 30%, caused by rapid development of drug resistance. Until now, the exact process of HGSC development is still obscure. Historically, the primary site of most epithelial ovarian cancers and in particular of HGSC was thought to be the ovarian epithelial surface. However, more recently it has been suggested that the cells that give rise to the majority of HGSC are from the fallopian tube fimbria (Karst et al. 2011; Quartuccio et al. 2016). These findings opened up new opportunities for early detection, prevention and treatment of ovarian cancer. Throughout the oncogenic process, the secretory cells of the Fallopian tubes retain PAX8 expression that possibly continues to exert its transcriptional activity on its physiological targets and may also function on newly available targets after the tumorigenic hits. Nevertheless, the role that PAX genes play in cancer is still unclear. However, it has been demonstrated that PAX8 could have important roles in the acquisition of the malignancy. In fact, the expression or overexpression of PAX proteins *per se* does not appear to be an initiating or a transforming molecular event in tumor pathogenesis, but could facilitate malignant development by controlling apoptotic resistance, tumor cell proliferation and migration, repression of terminal differentiation. The research group of Dr. Zannini in 2014 reported that PAX8 is involved in the tumorigenic phenotype of ovarian cancer cells. Specifically, they showed that PAX8 plays a critical role in migration, invasion and tumorigenic ability of ovarian cancer cells. In addition, PAX8 silencing strongly suppresses anchorage-independent growth *in vitro* and significantly inhibits tumorigenesis *in vivo* in a nude mouse xenograft model (Di Palma et al. 2014).

To understand the role of PAX8 in the oncogenic process beginning in Fallopian tube secretory cells and proceeding into metastatic epithelial ovarian cancer, we analyzed the transcriptome of normal and transformed cells upon PAX8 silencing. The results of our RNA-seq analysis strongly suggest that PAX8 could be involved in cell adhesion, migration and angiogenic pathways in both cell lines. Supporting this, in the second part of my PhD project, we demonstrate that PAX8 is indeed involved in the migration and adhesion of both primary human Fallopian tube secretory epithelial cells (Primary hFTSECs) and ovarian cancer cell lines (SKOV3, KURAMOCHI, OVSAHO and PEA1). In particular, we show that PAX8 silencing in Primary hFTSECs and ovarian cancer cells significantly affects the migration ability with respect to control cells. Moreover, upon PAX8 silencing in Primary hFTSECs and ovarian cancer cells display significantly less adherence when cultured on Fibronectin- or Collagen I-coated coverslips. Interestingly, Real-time qPCR analysis showed a significant downregulation of Integrin β_3 expression upon PAX8 silencing which correlates with the impairment of cell migration and adhesion in all cell lines used in this study.

Integrins are transmembrane glycoproteins that facilitate cell-cell and cell-extracellular matrix (ECM) adhesion and migration. They are obligate heterodimers that have two subunits: α (alpha) and β (beta). In mammals, 18 α and 8 β subunits have been identified, which combine to give 24 different αβ heterodimers (Hynes 2002). Upon ECM ligand binding, integrins are capable of modulating a variety of intracellular signal transduction cascades that mediate cellular signals such as regulation of the cell cycle, organization of the intracellular cytoskeleton and movement (Giancotti and Ruoslahti 1999; Kris A. DeMali et al. 2003). Integrin β_3 has been already implicated in a wide variety of functions, including platelet aggregation and thrombosis, implantation, placentation, angiogenesis, bone remodeling, and tumor progression. Integrin β_3 null mice show defects in angiogenesis, bone remodeling and homeostasis, with placental defects that lead to fetal mortality (Hodivala-Dilke et al. 1999). Integrin β_3 is able to pair with two subunits: α_v and α_{IIb}. Amongst integrins that have been identified as important mediators of ovarian cancer metastasis, the heterodimer Integrin $\alpha_v\beta_3$ holds a significant position. Integrin $\alpha_v\beta_3$ targeted either by antibodies or by small molecule inhibitors has been shown to inhibit

migration, adhesion, motility, angiogenesis, and proliferation in ovarian cancer cells *in vitro* (Carreiras et al. 2002; Markland et al. 2002; Leroy-Dudal et al. 2005).

To understand if PAX8 silencing could affect Integrin $\alpha_v\beta_3$ functional dimers in our cellular context, we performed an immunofluorescence using an antibody that specifically recognizes the heterodimer. It is interesting to note that $\alpha_v\beta_3$ heterodimer formation is entirely abrogated upon PAX8 silencing in both Primary hFTSEC and KURAMOCHI cells demonstrating the involvement of PAX8 in cell migration.

In conclusion, this study demonstrates that:
a) PAX8 silencing strongly affects the transcriptome of Fallopian tube secretory epithelial cells and ovarian cancer cells. Genes modulated by PAX8 in both cell types could be looked at as promising putative targets of this transcription factor whose function in this context is still unknown;
b) Retention of PAX8 from the initial hit in Fallopian tube secretory cells throughout the development of epithelial ovarian cancer occurs possibly because PAX8 confers a survival advantage to the cancer cells and maybe even drug resistance. Our results offer an interesting support for this selective advantage by demonstrating the regulatory role of PAX8 on Integrin β_3 and its functional dimer Integrin $\alpha_v\beta_3$.
It is important to highlight that this is the first study reporting the correlation between PAX8 and Integrin β_3 suggesting a possibly novel functional pathway in both normal and cancer context. We also demonstrate that PAX8 silencing affects the heterodimer Integrin $\alpha_v\beta_3$ reported to be used in integrin-based strategy to enhance tumor specific recognition of nanocarries and shown to be effective for several integrin inhibitors that are capable of blocking cancer progression (Kobayashi et al. 2017). Hence, this study is of great relevance because PAX8 targeted therapies for HGSC could be more promising and more specific than generic Integrin $\alpha_v\beta_3$ targeted therapies that acquire drug resistance over time.

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