Candidate genes and pathways downstream of PAX8 involved in ovarian high-grade serous carcinoma

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ABSTRACT

Understanding the biology and molecular pathogenesis of ovarian epithelial cancer (EOC) is key to developing improved diagnostic and prognostic indicators and effective therapies. Although research has traditionally focused on the hypothesis that high-grade serous carcinoma (HGSC) arises from the ovarian surface epithelium (OSE), recent studies suggest that additional sites of origin exist and a substantial proportion of cases may arise from precursor lesions located in the Fallopian tubal epithelium (FTE). In FTE cells, the transcription factor PAX8 is a marker of the secretory cell lineage and its expression is retained in 96% of EOC. We have recently reported that PAX8 is involved in the tumorigenic phenotype of ovarian cancer cells. In this study, to uncover genes and pathways downstream of PAX8 involved in ovarian carcinoma we have determined the molecular profiles of ovarian cancer cells and in parallel of Fallopian tube epithelial cells by means of a silencing approach followed by an RNA-seq analysis. Interestingly, we highlighted the involvement of pathways like WNT signaling, epithelial-mesenchymal transition, p53 and apoptosis. We believe that our analysis has led to the identification of candidate genes and pathways regulated by PAX8 that could be additional targets for the therapy of ovarian carcinoma.

INTRODUCTION

Ovarian cancer, like other cancers, is a spectrum of diseases and not a single disease entity [1]. Malignant surface epithelial tumors (carcinomas) are the most common ovarian cancers, accounting for 90% of the cases. These tumors are divided in four major histotypes: serous, mucinous, endometrioid and clear cell. Of these, high-grade serous carcinoma (HGSC) is the most common and lethal histotype, associated with abnormalities of BRCA1, BRCA2 and p53 [2]. This cancer is extremely genetic unstable and heterogenous; it is rarely detected while confined to the ovary and in about 90% of the cases it is diagnosed when women have already intra-abdominal spread. Although research has traditionally focused on the hypothesis that HGSC arises from the ovarian surface epithelium (OSE), it has been recently suggested that it may arise from the Fallopian tube fimbria. In particular, the serous tubal intraepithelial carcinomas (STICs), which are identified in the distal end (fimbria) of the Fallopian tube and arise from p53 mutations, have been indicated as the primary lesions that evolve through subsequent oncogenic events into HGSC [3]. This hypothesis is further supported by genetically engineered murine models that mimic the transformation from Fallopian tubal secretory epithelial cells to HGSC [4].

The transcription factor PAX8 is a marker of the Fallopian tube secretory cell lineage and its expression is retained in 96% of the serous ovarian carcinomas, in 89% of the endometrioid and 100% of the clear cell carcinomas, whilst it is not detected in the mucinous carcinoma [5]. PAX8 is a member of the PAX (PAired boX) gene family, genes tightly regulated in both temporal and spatial expression patterns [6]. PAX8 is crucial for the organogenesis of the thyroid gland, kidney, nervous system and Mullerian system [7, 8]. In the adult, PAX8 is expressed in the thyroid gland, in the renal excretory system and in tissues derived from the Mullerian ducts.
Our recent results demonstrated that PAX8 plays a critical role in cell cycle progression and cell survival of differentiated epithelial cells [11], reinforcing the involvement of this transcription factor in different biological processes. In tumors, PAX8 is involved in the progression of follicular thyroid carcinomas [12] and it is overexpressed in the majority of gliomas, Wilms tumors and well-differentiated pancreatic neuroendocrine tumors [10, 13, 14]. In the scenario of ovarian cancer, PAX8 is the currently available most important marker [15] being a useful IHC target for the diagnosis of Mullerian tumors [16]. Despite PAX8 is normally expressed in Fallopian tube secretory cells, namely the cell of origin of HGSC, and is mainly considered a marker of tumor origin, we have recently reported its pivotal function in the tumorigenic phenotype of ovarian cancer cells. In particular, we showed that PAX8 plays a critical role in the migration, invasion and tumorigenic ability of ovarian cancer cells. In our published study, PAX8 silencing strongly suppressed anchorage-independent growth in vitro and significantly inhibited tumorigenesis in vivo in a nude mouse xenograft model [17]. In addition, the Cancer Genome Atlas (TGCA) Project indicated PAX8 as a survival gene essential for the proliferation of ovarian cancer cells [5]. Overall, PAX8 belongs to a class of lineage-survival genes that are required for both normal development of specific tissues and for cancer cell proliferation/survival.

On the basis of such evidences, it is conceivable that PAX8 is intimately involved in the progression of ovarian carcinomas. However, its function in Fallopian tube secretory epithelial cells, as well as the entire molecular network that allows PAX8 to regulate cellular processes in these cells and in ovarian cancer cells, is still an unexplored field. To improve our knowledge of the complex role of this transcription factor, we thought that it would be beneficial to uncover the downstream gene network governed by this transcriptional factor both in Fallopian tubal epithelium and ovarian cancer cells. Hence, the aims of our study were (1) to compare the transcriptome of normal Fallopian tube secretory epithelial cells with that of ovarian cancer cells in order to uncover genes and pathways modified during the transformation process; (2) to investigate PAX8 downstream gene regulatory network in physiological and pathological conditions to identify genes and pathways regulated by PAX8 that could be additional targets for the therapy of ovarian carcinoma.

RESULTS

Identification of genes differentially expressed between FT194 and SKOV-3 cells

Primary questions in the field of ovarian cancer biology concern its developmental cell of origin. The epithelial cells covering the ovaries (OSE) have historically been considered the site of origin of all ovarian cancers but recent evidence suggests that high-grade serous carcinoma (HGSC) originates from the Fallopian tube (FT) epithelium or the tuboperitoneal junction rather than from the OSE [4, 18].

To identify genes differentially expressed in the Fallopian tubal epithelium and in ovarian carcinoma and to disclose some of the pathways that might contribute to the carcinoma formation, an RNA sequencing analysis was performed on FT194 Fallopian tube secretory epithelial cells and SKOV-3 ovarian adenocarcinoma cells and a total of 12628 genes were mapped to the reference human genome (Supplementary Table S1). Of these, 7451 genes were differentially expressed in the two cell types at significant level (FDR-adjusted p-value ≤ 0.05) (Supplementary Table S2): 3553 genes resulted more expressed in SKOV-3 cells than in FT194 cells and 3778 genes were found less expressed in SKOV-3 cells with respect to FT194 cells. Interestingly, 38 genes were exclusively expressed in SKOV-3 cells, while 82 genes were present only in FT194 cells (Supplementary Table S2).

To date, despite the identification of a number of key mutations in p53 and BRCA1/2 genes, the complexity of the molecular pathway(s) underlying epithelial ovarian cancer has not been yet fully elucidated. To focus our attention on genes showing the greatest expression differences between FT194 and SKOV-3 cells, we applied a 2 fold-change cutoff and we searched for enrichment of specific gene sets at the Molecular Signatures Database (MsigDB). In particular, we explored the hallmark gene sets that summarize and represent specific well-defined biological states or processes. As shown in Figure 1A, genes up-regulated ≥ 2 fold in SKOV-3 cells present a significant enrichment for pathways such as estrogen response, epithelial mesenchymal transition, response to UV DNA damage, angiogenesis and Wnt/β-catenin signaling. There are numerous evidences, both in vitro and in vivo studies, that estrogens might regulate ovarian carcinogenesis [19]. It is well known that hormones stimulate the proliferation of the cells, meaning that they might function as carcinogens. For example, in ovarian cancer cells 17β-estradiol (E2) increases the ROS and NO production that participates in cancer progression [20]. In addition, aberrant Wnt/β-catenin signaling is implicated in several cancers including epithelial ovarian cancer [21, 22] and it represents one of the pathway involved in epithelial-mesenchymal transition (EMT) responsible for cancer cell dissemination and metastasis formation [23]. Stressful conditions such as UV radiations, hypoxia, and alterations associated with changes in cell phenotype that include EMT as well as angiogenesis represent main processes that provide carcinogenesis. At the same time, genes down-regulated ≥ 2 fold in SKOV-3 cells (Figure 1B) are involved in pathways as TNF-α signaling, IFN-α and IFN-γ responses and inflammatory response. TNF-α, IFN-α and IFN-γ are pleiotropic cytokines
Figure 1: MsigDB analysis of the most relevant genes differentially expressed between FT194 and SKOV-3 cells. (A) MsigDB for genes upregulated ≥ 2 fold in SKOV-3 cells. (B) MsigDB for genes downregulated ≥ 2 fold in SKOV-3 cells.
with diverse physiological functions such as activation of macrophages, induction of immune response and apoptosis and inhibition of cell proliferation. It has been reported that in ovarian cancer the above-mentioned cytokines exert an anti-proliferative activity and induce apoptosis [24, 25]. Eventually, if the immune response might contribute to the tumorigenic process, it may be also helpful in preventing the tumor properties.

Among the 38 genes exclusively expressed in SKOV-3 cells (Supplementary Table S2), ALDH1A1 [26], GABRA3 [27], FOLR1 [28], DYSPLS5 [29], CGB8 [30], C8orf4 [31] and MAGEB2 [32] could be intriguing for the development and maintenance of the neoplastic phenotype. Similarly, among the 82 genes expressed only in FT194 cells (Supplementary Table S2) CDKN2A [33], MT1G [34], GPX7 [35], HCK [36], ZBTB16 [37] could be looked at as interesting genes being tumor suppressor epigenetically silenced in cancer cells.

**PAX8 downstream target genes in FT194 and SKOV-3 cells**

As already mentioned, in the Fallopian tube epithelium the transcription factor PAX8 is a marker of the secretory cell lineage. Our RNA sequencing analysis performed on FT194 Fallopian tube secretory epithelial cells and SKOV-3 ovarian adenocarcinoma cells confirmed that PAX8 is expressed at similar levels in these two cell types (Supplementary Table S2), supporting the hypothesis that PAX8 is not overexpressed in epithelial ovarian carcinoma but rather that its expression is conserved from the suggested cell of origin [38]. Recently, we have provided the first evidence of a clear involvement of PAX8 in the in vivo tumorigenesis of ovarian cancer cells [17], reinforcing the role of PAX genes in cancer through their effects on apoptosis resistance, tumor cell proliferation and migration, and repression of terminal differentiation.

On the basis of our previous data and in order to clarify PAX8 contribution to ovarian cancer through the identification of its downstream gene regulatory network, we have transiently knocked-down PAX8 expression in both FT194 and SKOV-3 cells. We chose to inhibit PAX8 expression also in the FT194 cell line because to date its role in the physiological contest of the Fallopian tube secretory cell has not been investigated. Moreover, we reasoned that genes affected by PAX8 silencing in both cell lines could be looked at as PAX8 targets with greater confidence.

Three independent silencing experiments were performed and analyzed by RNA-seq unraveling a total of 467 genes modulated by PAX8 expression in SKOV-3 and FT194 cells (Supplementary Table S3 and Supplementary Table S4). The reduced expression of PAX8 in the samples was confirmed by the RNA-seq findings, which showed an 80% decrease (FDR-adjusted \( p \)-value \( \leq 0.05 \)) of which 214 were downregulated while 87 resulted upregulated (Supplementary Table S3). As shown in Table 1, among the most downregulated genes based on FDR there are TUBB2B, PPME1, ATP8A1, USP2, BLCAP, DIAPH1, FGF18 and KIF12.

**TUBB2B** (tubulin beta 2B class Ib) may have important roles in tumor progression and chemoresistance [39]. **PPME1** is a protein phosphatase 2A (PP2A)-specific methylesterase that mediates the demethylation and inactivation of PP2A. This protein is a human tumor suppressor that accounts for the majority of cellular serine/threonine phosphatase activity [40]. **ATP8A1** is a member of P4-ATPase family with a role in the formation of membrane ruffles to promote cell migration [41]. **BLPAP** (Bladder cancer-associated protein) is a highly conserved protein among species displaying tissue-specific expression patterns, but also tissue-specific functions being both a tumor suppressor and a tumor promoting [42]. Recent data revealed that the blockade of DIAPH1-tubulin interaction might be a promising approach to inhibit one of the earliest steps in the metastatic cascade of colon cancer [43]. **FGF18** is a pleiotropic growth factor involved in skeletal growth and development [44, 45]. Deregulated FGF signaling may affect some oncogenic mechanisms and properties in various tumor types [46]. **KIF12** belongs to the kinesin superfamily of motor proteins that bind microtubules and mediate the intracellular transport of organelles and protein complexes [47].

Interestingly, in siPAX8-SKOV-3 cells among the most up-regulated genes based on FDR there are DKK1 and **BOD-1** [48] (Table 1). DKK1 belongs to the Dickkopf gene family, which encodes secreted glycoproteins to control cell fate and neural patterning during embryonic development. It was first identified as an inhibitor of the Wnt signaling pathway. **BOD-1** is a member of the FAM44 protein family and is highly conserved throughout metazoans. Depletion of **BOD-1** in human cells causes severe biorientation defects [49].

In FT194 cells, 166 genes affected by PAX8 silencing reached genome-wide significance after correction for multiple testing (FDR-adjusted \( p \)-value \( \leq 0.05 \)) of which 119 were downregulated while 47 were upregulated (Supplementary Table S4). Among the most significantly downregulated genes there are: **FGF18**, **CDH6**, **CHRDL**, **ZBED2**, **CNTN4**, **ANXA2** (Table 2). Very interestingly, **FGF18** results among the top 20 downregulated genes upon silencing of PAX8 in both SKOV-3 and in FT194 cells (Table 1 and Table 2).

**CDH6**, a member of the cadherin superfamily, is required for the kidney development as well as ganglia formation [50, 51]. Increased expression of this gene may be associated with tumor growth and metastasis [52]. **CHRDL** functions as a dorsalizing factor for early vertebrate
Table 1: Differentially expressed genes that are significantly modulated after PAX8 silencing in SKOV-3 cells

| Gene Name | NCBI gene ID | FPKM1 | FPKM2 | Log₂ fold change | FDR q value | Fold change |
|-----------|--------------|-------|-------|------------------|-------------|-------------|
| ANKRD34B  | 340120       | 0.517453 | 2.34955 | 2.18288          | 0.00400937 | 4.54059     |
| PAX8      | 7849         | 8.65929  | 30.224  | 1.80338          | 0.00400937 | 3.49037     |
| TUBB2B    | 347733       | 0.483395 | 1.54683 | 1.67804          | 0.015208   | 3.19993     |
| PPME1     | 51400        | 11.285   | 34.0561 | 1.59351          | 0.00400937 | 3.01783     |
| LMLN      | 89782        | 0.558739 | 1.61854 | 1.53445          | 0.00400937 | 2.89678     |
| H2AFY2    | 55506        | 6.68489  | 19.0865 | 1.51358          | 0.00400937 | 2.85518     |
| PHTF2     | 57157        | 3.23708  | 8.76508 | 1.43287          | 0.00400937 | 2.70770     |
| DCAF12L1  | 139170       | 1.66899  | 4.506   | 1.43287          | 0.00400937 | 2.69983     |
| GRIN2B    | 2904         | 0.727982 | 1.94915 | 1.42087          | 0.0127719  | 2.45920     |
| EPRS      | 2058         | 18.5003  | 47.5384 | 1.36154          | 0.00400937 | 2.56959     |
| PPAP2B    | 10904        | 9.66632  | 23.4671 | 1.29819          | 0.00400937 | 2.42771     |
| ATP8A1    | 304          | 0.582276 | 1.43193 | 1.29819          | 0.0127719  | 2.42771     |
| USP2      | 113220       | 2.32013  | 5.50046 | 1.24535          | 0.00400937 | 2.37076     |
| C10orf46  | 143384       | 14.0943  | 33.1881 | 1.23555          | 0.00400937 | 2.35471     |
| ANXA2P2   | 100507412    | 362,087  | 11,467  | 1.08783          | 0.00400937 | 1.08783     |
| PSAP      | 5660         | 201.624  | 467.609 | 1.21364          | 0.00400937 | 2.31922     |
| DIO2      | 1734         | 5.30475  | 1.69721 | 1.064412         | 0.00400937 | 3.12557     |
| ROS1      | 6098         | 2.97713  | 1.06039 | 1.48933          | 0.00400937 | 2.80759     |
| P4HA3     | 283208       | 1.91889  | 0.746284 | 1.36248        | 0.00400937 | 2.57127     |
| PTPMT1    | 114971       | 14.4382  | 5.88494 | 1.29426          | 0.0196014  | 2.45251     |
| MET       | 4233         | 47.8695  | 20.5651 | 1.21891          | 0.00400937 | 2.32771     |
| CCIN      | 881          | 1.24831  | 0.56605 | 1.14109          | 0.0361254  | 2.20548     |
| ARTN      | 9048         | 5.77216  | 2.63816 | 1.12958          | 0.00400937 | 2.18795     |
| LOC100507412 | 100507412 | 394,087  | 186.2   | 1.08166          | 0.00400937 | 2.11647     |
| SERPINB5  | 5268         | 1.49223  | 0.722489 | 1.04642       | 0.0340659  | 2.06540     |
| SERPINE1  | 5054         | 128.607  | 62.4787 | 1.04153          | 0.00400937 | 2.05841     |
| DKK1      | 22943        | 85.9562  | 42.0833 | 1.03035          | 0.00400937 | 2.04252     |
| SGK1      | 6446         | 18.4838  | 9.10546 | 1.02146          | 0.00400937 | 2.02997     |
| FGF1      | 2246         | 1.67617  | 0.843698 | 0.990371      | 0.00400937 | 1.98670     |
| BOD1      | 91272        | 27.4212  | 14.2379 | 0.945548         | 0.00400937 | 1.92592     |
| CCND3     | 896          | 27.6423  | 14.3971 | 0.941101         | 0.00400937 | 1.91999     |
| TGFBI     | 7042         | 13.4826  | 7.11507 | 0.922151         | 0.00400937 | 1.89494     |
| CALU      | 813          | 57.1015  | 30.1837 | 0.919757         | 0.00400937 | 1.89180     |
| ANKRD1    | 27063        | 10.4157  | 5.56174 | 0.905149         | 0.00400937 | 1.87274     |
| CGB8      | 94115        | 11.0552  | 5.93536 | 0.897321         | 0.015208   | 1.86260     |
| C6orf120  | 387263       | 18.0817  | 9.71162 | 0.896746         | 0.00400937 | 1.86186     |

Note: FPKM1 and FPKM2 indicate fragments per kilobase of exon per million mapped reads after and before PAX8 silencing, respectively. Genes from 1 to 20 are downregulated and genes from 21 to 40 are upregulated.
embryonic tissues by binding to ventralizing TGF-beta family bone morphogenetic proteins (BMPs) and sequestering them in latent complexes [33]. ZBED2 (Zinc Finger, BED-Type Containing 2) is a member of family factors involved in the regulation of various functions in vertebrates [54]. CNTN4 is a glycosylphosphatidylinositol (GPI)-anchored neuronal membrane protein responsible for the formation of axon connections in the developing nervous system [55]. Loss of heterozygosity (LOH) and gene sequencing analysis investigate the possibility of CNTN4 to function as tumor suppressor gene in ovarian cancer [56]. ANXA2 is a calcium-binding cytoskeletal protein aberrantly expressed in a wide spectrum of cancers and in EOC may promote cell proliferation [57].

Additionally, in siPAX8-FT194 cells among the top 20 significantly upregulated genes there are: SERPINB2, FPR1, PAPPA, CCL20, RHOB, DCN (Table 2).

SERPINB2, also named Plasminogen Activator Inhibitor, Type II (PAI-2) is upregulated by numerous inflammatory conditions [58]; in cancer disease its expression is often an indicator of positive prognosis as described in ovarian cancer [59, 60]. FPR1 is a G protein-coupled receptor that promotes growth, angiogenesis and invasion in glioblastoma tumor [61]. FPR1 as the other two FPRs play a pivotal role in inflammatory response, tissue repair, tumor growth, physiological and pathological angiogenesis [62]. PAPPA (pregnancy-associated plasma protein A) regulates mitotic progression through modulating the IGF1 signaling pathway [63]. CCL20 is a small cytokine constitutively produced by Fallopian tube epithelial cells and able to function as endogenous anti-viral microbicidal of female reproductive tract [64]. In ovarian cancer, CCL20 is one of the primary chemokine induced via NF-kB pathway [65]. RHOB, is a member of small GTPases belonging to the Ras protein superfamily, might have a suppressive activity in cancer progression, in fact its loss occurs frequently in ovary carcinogenesis and progression. Moreover, ectopic expression of Rhob into nude mice is highly effective in suppressing tumor growth of ovarian cancer xenografts [66]. DCN is a small leucine-rich proteoglycan, component of connective tissue responsible for the matrix assembly. Also this factor might function as a tumor suppressor in ovarian cancer, because its expression is lost during epithelial transformation [67].

**PAX8 regulates the expression of shared and exclusive sets of genes in SKOV-3 and FT194 cells**

As illustrated in the above paragraph, following PAX8 silencing 214 downregulated and 87 upregulated genes were identified in siPAX8-SKOV-3 cells while 119 downregulated and 47 up-regulated were defined in siPAX8-FT194 cells (FDR-adjusted p-value ≤ 0.05) (Supplementary Table S3 and Supplementary Table S4). A Venn diagram was constructed to identify shared and distinct sets of genes regulated by PAX8 in the two cell types. As shown in Figure 2A, 47 genes are commonly downregulated in siPAX8-SKOV-3 and siPAX8-FT194 cells, whereas the genes downregulated only in one of the samples are 167 and 72 (SKOV-3 and FT194, respectively). Analogously, 15 genes are commonly upregulated in siPAX8-SKOV-3 and siPAX8-FT194 cells while 72 and 32 genes are exclusively upregulated in SKOV-3 and FT194 cells, respectively (Figure 2B). The complete lists of the genes classified according to the Venn diagram are shown in Table 3.

We reasoned that genes modulated upon PAX8 silencing in both cell lines could be looked at as putative PAX8 targets with greater confidence. Hence, we focused our attention on the intersections between SKOV-3 and FT194 cells and we observed that they include several interesting genes such as: PSAP, FGF18, CDH6, ROR1, RBPI and DNMT3B whose role in cell proliferation, cell survival and tumorigenic process has already been reported. In particular, PSAP (prosapson) is a pleiotropic growth factor able to prevent cell death or apoptosis and to promote cell survival [68]; FGF18 controls migration, invasion and tumorigenicity of ovarian cancer cells [69]; CDH6 is a new TGF-beta target modulated as a mesenchymal marker in EMT [52]; ROR1, a receptor tyrosine kinase orphan receptor 1, is involved in migration, invasion and EMT in ovarian cancer cells [70]; RBPJ (recombination signal-binding protein Jk) is a key transcription factor in the Notch signaling pathway and its inhibition reduces cell growth [71]; DNMT3B encodes a DNA methyltransferase involved in de novo DNA methylation whose role in cancer development is not clear but several reports suggest a role as tumor suppressor [72].

At the same time, we believe that genes modulated upon PAX8 silencing exclusively in FT194 or in SKOV-3 cells should be considered relevant because they could reflect the continuous process from the precancerous to the cancerous condition. In addition, the tumorigenic process might itself promote the expression of some genes making them available for PAX8 transcriptional regulation. For example, WNT7A known to function as tumor-promoting in ovarian cancer [73] is present at very low level in FT194 cells but becomes highly expressed in SKOV-3 cells (Supplementary Table S1) where is significantly downregulated upon PAX8 silencing (Supplementary Table S3 and Table 3A). Similarly, ASRGL1 (asparaginase like 1) is highly expressed in ovarian carcinoma and confers a selective growth advantage [74]; accordingly, our data show that ASRGL1 is preferentially expressed in SKOV-3 cells where it is downregulated following PAX8 silencing (Table 3A).

We validated the data obtained by RNA-seq by means of qRT-PCR for 13 genes, including PAX8. For these genes, we confirmed the different expression between the two cell types (Figure 3A) and in OVCAR-3, PEA1 and PEO14 ovarian cancer cell lines (Supplementary Figure S1). The
Table 2: Differentially expressed genes that are significantly modulated after PAX8 silencing in FT194 cells

| Gene Name | NCBI gene ID | FPKM1 | FPKM2 | Log₂ fold change | FDR q value | Fold change |
|-----------|--------------|-------|-------|------------------|-------------|-------------|
| 1 CDH6    | 1004         | 0.904512 | 5.66947 | 2.648 | 0.00642248 | 6.26798 |
| 2 SNORA26 | 677810       | 12.5059 | 73.4428 | 2.55401 | 0.00642248 | 5.87264 |
| 3 FGF18   | 8817         | 2.9921  | 14.7197 | 2.29852 | 0.00642248 | 4.91953 |
| 4 CHRD    | 8646         | 1.14353 | 5.39776 | 2.23887 | 0.00642248 | 4.72027 |
| 5 KLHL14  | 57565        | 0.218892 | 0.999955 | 2.19164 | 0.00642248 | 4.56824 |
| 6 EPH1    | 2047         | 0.17794 | 0.77373 | 2.12044 | 0.0162802 | 4.34827 |
| 7 ZBED2   | 79413        | 0.98563 | 4.13057 | 2.06722 | 0.00642248 | 4.19078 |
| 8 TLL2    | 7093         | 0.625627 | 2.60957 | 2.06043 | 0.00642248 | 4.17111 |
| 9 PAX8    | 7849         | 9.23106 | 33.8269 | 1.8736  | 0.00642248 | 3.66446 |
| 10 BAALC  | 79870        | 0.360111 | 1.28504 | 1.8353  | 0.0333357 | 3.56846 |
| 11 MAL     | 4118         | 7.14709 | 25.0391 | 1.80876 | 0.00642248 | 3.50341 |
| 12 DCDC2  | 51473        | 4.33336 | 14.9979 | 1.7912  | 0.00642248 | 3.46103 |
| 13 LOC643201 | 643201       | 1.32389 | 4.51658 | 1.77045 | 0.00642248 | 3.41160 |
| 14 ADAMTS14 | 140766        | 0.900768 | 3.0108  | 1.74092 | 0.00642248 | 3.34248 |
| 15 CNTN4  | 152330       | 0.800182 | 2.67321 | 1.74017 | 0.00642248 | 3.34075 |
| 16 ANXA2   | 302          | 572.227 | 1838.55 | 1.68391 | 0.00642248 | 3.21298 |
| 17 FAT2    | 2196         | 0.373409 | 1.18513 | 1.66622 | 0.00642248 | 3.17382 |
| 18 ANXA2P2 | 304          | 1.12435 | 3.55187 | 1.65948 | 0.0115711 | 3.15903 |
| 19 ROR1    | 4919         | 7.26579 | 22.3551 | 1.62141 | 0.00642248 | 3.07676 |
| 20 LMLN    | 89782        | 0.291647 | 0.876636 | 1.58775 | 0.0115711 | 3.00580 |
| 21 SERPINB2 | 5055         | 4.52656 | 1.12888 | -2.00352 | 0.00642248 | 4.00977 |
| 22 FPR1    | 2357         | 18.91   | 7.18704 | -1.39568 | 0.00642248 | 2.63113 |
| 23 PAPPA   | 5069         | 4.39506 | 1.94254 | -1.17794 | 0.00642248 | 2.26253 |
| 24 PTGS2   | 5743         | 14.4361 | 6.46952 | -1.15795 | 0.00642248 | 2.23140 |
| 25 NRK     | 203447       | 2.78453 | 1.25142 | -1.15387 | 0.00642248 | 2.22510 |
| 26 ST6GALNAC5 | 81849         | 10.3815 | 4.81544 | -1.10827 | 0.00642248 | 2.15587 |
| 27 THBD    | 7056         | 7.82686 | 3.66541 | -1.09446 | 0.00642248 | 2.13533 |
| 28 CCL20   | 6364         | 60.3995 | 30.2135 | -0.999342 | 0.0162802 | 1.99909 |
| 29 RHOB    | 388          | 44.0161 | 22.2603 | -0.983562 | 0.00642248 | 1.97734 |
| 30 F3      | 2152         | 132.89  | 67.4915 | -0.977451 | 0.00642248 | 1.96989 |
| 31 TGFβ2   | 7042         | 53.4068 | 27.4917 | -0.958027 | 0.00642248 | 1.94265 |
| 32 ZCCHC2  | 54877        | 9.21038 | 4.7504  | -0.955212 | 0.00642248 | 1.93886 |
| 33 DCN     | 1634         | 20.2125 | 10.5292 | -0.940855 | 0.00642248 | 1.91967 |
| 34 MX2     | 4600         | 66.0911 | 34.8487 | -0.923353 | 0.00642248 | 1.89652 |
| 35 RSAD2   | 91543        | 72.3042 | 38.7711 | -0.899096 | 0.00642248 | 1.86490 |
| 36 MSRB3   | 253827       | 35.8124 | 19.2352 | -0.896709 | 0.00642248 | 1.86181 |
| 37 MET     | 4233         | 54.6992 | 30.1665 | -0.858572 | 0.00642248 | 1.81324 |
| 38 MX1     | 4599         | 264.614 | 148.11  | -0.837216 | 0.00642248 | 1.78660 |
| 39 CYP1B1  | 1545         | 64.4002 | 36.0841 | -0.835702 | 0.00642248 | 1.78473 |
| 40 NCOA7   | 135112       | 27.7387 | 15.6627 | -0.824561 | 0.00642248 | 1.77100 |

Note: FPKM1 and FPKM2 indicate fragments per kilobase of exon per million mapped reads after and before PAX8 silencing, respectively. Genes from 1 to 20 were downregulated and genes from 21 to 40 were upregulated.
Table 3A: List of shared and exclusive genes downregulated upon PAX8 silencing in SKOV-3 and FT194 cells

| Name     | Total | Elements                                                                 |
|----------|-------|--------------------------------------------------------------------------|
| SKOV3-FT194 | 47    | NUP35, ANXA2, HDGFRP3, STX3, DUSP11, AP1G1, ENPP4, PHLDA3, PSAP, ENPP1, EPR5, RHPN2, C10orf46, GPR63, CDK2AP1, H2AFY2, PPME1, LMLN, KITLG, CDH6<sup>a</sup>, FAM107B, C1orf186, BLCAP, PRKAA2, KCNQ1, APROP, ADCY9, AMY2A, KCNQ3, ADCY9, ASONA1, NPTN, MINT4, ANG, NCOA7, PDK1, PDK3, BCSK1, GPR150, GPR154, NDUFAF3, TP53, TP63, TP73, KLF14, LOC100507412, PCSK7, CPA4, CIRBP, SERPINE1, C6orf120, PODXL, CCDC80, DOCK2, CLEC2D, HIRA, C3orf49, LASS3, C3orf43, C3orf42, C3orf41, C3orf40, C3orf39, C3orf38, C3orf37, C3orf36, C3orf35, C3orf34, C3orf33, C3orf32, C3orf31, C3orf30, C3orf29, C3orf28, C3orf27, C3orf26, C3orf25, C3orf24, C3orf21, C3orf20, C3orf18, C3orf17, C3orf16, C3orf15, C3orf14, C3orf13, C3orf12, C3orf11, C3orf10, C3orf9, C3orf8, C3orf7, C3orf6, C3orf5, C3orf4, C3orf3, C3orf2, C3orf1, C2orf72, RRM2, BCAT1, SLC7A11, ADAMTS9, TMSB4X, TPK1, KPNA6, ATP8A2, CHST12, THBS1, CYP4F11, RNF145, ADAMTS5, FLJ26245, SYTL2, UBE2H, DCAF12L1, RAB11FIP2, ABI2, PTPRB, FIGN, PFAH1B2, TCF12, PMAIP1, WDR44, DCUN1D3, SLC30A6, EPHA4, NEBL, DSC2, CNOT6, ZNF611, MYO10, MAP2, SLC17A6, ADAM10, CBS, KIF12, KLHL13, ZNF185, PPARGC1A, WASF1, PLAU, KLHL14, TLL2, CHRD, CLGN, NGFR, ARHGFE37, FLG, GAS7, TGFB3, RGS20, ILD2R, SPON1, MMD, CD24, AP1M2, TNFSF4, THY1, ADAMTS14, ZBED2<sup>b</sup>, PLCB4, ADAMTS1, LOC643201, NOV, MPP7, SHISA2, ST3GAL1, SOX17, GDF6, ANK3, RASGEF1B, CNTN4, C10orf15, SULF2, ADORA1, MEGF9, KLKR2, ITGB8, DANC4, SNORA26, SLC6A6, BAALC, CTHRC1, G0S2, TMEM117, AIF1L, GJB2, RPRKAG2, FAT2, MST1, DCDC2, OCTR, SDC2, INHBB, KIAA1456, BTBD11, CA2, NID2, SLC47A1, CDH5, PDE1A, COL12A1, KCTD5, RCBTB2, CANT1, MTPN, FN1, UBE2D1, ZCCHC4, TMC2, TRIM24, FAM174B, C10orf26, TMCO7, GUCY1B3, ARHGFE1, ANKR52D, RC3H2, ZNF618, FIH1, TP1, PTPN13, DCN, PAPPAb, RHOB, CCL20, GBP1, SERPINB2, OA53, PDZD2, IFI19, NCOA7, OA52, RASD2, PTGS2, MX1, ZCCHC2, CYP1B1, DHX58, DDX60L, HMGA2, FAR2P, MX2, NKR, FPR1, THBD, ST6GALNAC5, THBS2 |

<sup>a</sup>Genes that were validated using qRT-PCR.

Table 3B: List of shared and exclusive genes upregulated upon PAX8 silencing in SKOV-3 and FT194 cells

| Name     | Total | Elements                                                                 |
|----------|-------|--------------------------------------------------------------------------|
| SKOV3-FT194 | 15    | ANKR1D1, F3, G3BP2, TGBF2, MAPK1IP1L, TOM20, MBTPS1, PTPN1, MSR8B3, MET, RBPJ, TCEB3, WDR1, STX12 |
| SKOV3    | 72    | DIO2<sup>b</sup>, ZCCHC3, NTN4, NGRN, TRAPPC2, CALU, MOK, DCBLD1, PTPMT1, GFG1, C1D, AHR, PDCD6, LOC50057412, RN455, PCSK7, CPA4, CIRBP, SERPINE1, C6orf120, PODXL, CCDC80, DOCK10, PGY2, P4HA3, MCFD2, DSEL, MYCN, LPXN, CCND3, CHFR, MAP2K4, GRWD1, CCBE1, UBE2G1, ZFEB1, BOD1<sup>b</sup>, NCF2, ODZ2, ERRFI1, CCIN, SERPINB5, ATG12, CBG8, FLRT2, AIM1, ROS1, C7orf58, ALS2CL, FRMD6, KRT5, SSFA2, PIP4KA2, SOCS7, NT5E, ESYT2, SMAP1, ARTN, SCRN1, DKK1, EFEMP1, RFK, SGK1, CCDC68, CRK, FOXL1, PGM2L1, PCDH10, SEMA7A, ZFAND3, IFI02, CCNC, RAN1, KINHYN, POLR3F |
| FT194    | 32    | CMPK2, RAD54L2, DDX58, IFIT1, SP110, PTPN13, DCN, PAPPAb, RHOB, CCL20, GBP1, SERPINB2, OA53, PDZD2, IFI19, NCOA7, OA52, RASD2, PTGS2, MX1, ZCCHC2, CYP1B1, DHX58, DDX60L, HMGA2, FAR2P, MX2, NKR, FPR1, THBD, ST6GALNAC5, THBS2 |

<sup>b</sup>Genes that were validated using qRT-PCR.
same genes were also validated upon silencing of PAX8 in FT194 and SKOV-3 cells (Figure 3B and 3C). In particular, we confirmed that KIF12, DIO2 and WNT7A are expressed preferentially in SKOV-3 cells (Figure 3A) and in these cells are modulated upon silencing of PAX8 (Figure 3B). At difference, FGF18, CDH6, ANXA2 and ROR1 are expressed and modulated in both cell lines (Figure 3A, 3B, 3C). CHRD, PAPPA and SERPINB2 are expressed almost exclusively in FT194 cells (Figure 3A) and are modulated upon silencing of PAX8 (Figure 3C). ZBED2 and BOD1 are expressed in both cell lines but following PAX8 silencing ZBED2 is modulated at significant level only in FT194 cells, while BOD1 only in SKOV-3 cells. To strengthen our observations we validated the same genes also in PEA1 cells after PAX8 silencing (Supplementary Figure S2).

Subsequently, we analyzed the 5′-flanking regions of the genes commonly regulated in the two cell lines in order to recognize DNA binding motifs for PAX8 matrices using the PASTAA method (http://trap.molgen.mpg.de) [75] which utilizes the prediction of binding affinities of a transcription factor to regulatory regions. The genes containing PAX8 DNA binding motifs were ranked according to the prediction of binding affinity of their 5′-flanking region to the PAX8 binding sites (Supplementary Table S5). To unambiguously determine whether PAX8 directly binds to the above mentioned regulative genomic sequences, we performed a computational analysis for some representative genes chosen among those commonly regulated in the FT194 and SKOV3 cells, using the MatInspector Software (Genomatix). We searched for PAX8 binding sites in a region of about 2 Kb in their 5′-flanking region and we found several PAX8 consensus sequences. To confirm the predictions of the MatInspector analysis, we carried out chromatin immunoprecipitation (ChIP) assays on FT194 cells using a polyclonal antibody against PAX8. The ChIP results indicate that in vivo PAX8 is able to bind the regulatory regions of all selected genes (Figure 4).

All together, our results show that the expression profiles affected by PAX8 silencing might reflect an ongoing transformation process reinforcing the involvement of this transcription factor in ovarian cancer.

Pathways regulated by PAX8 in ovarian cancer

The pathways that PAX8 may regulate in ovarian cancer and in Fallopian tube secretory cells are still undefined. To categorize PAX8 associated pathways that are represented in our PAX8-silenced SKOV-3 and FT194 cells, we classified all the dysregulated genes (301 for SKOV-3 and 166 for FT194) using the Gene

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**Figure 2:** The Venn diagram of genes modulated upon PAX8 knockdown in SKOV-3 and FT194 cells. (A) Overlap and differences of downregulated genes following PAX8 silencing between SKOV-3 and FT194 cells. (B) Overlap and differences of upregulated genes following PAX8 silencing between SKOV-3 and FT194 cells.
Figure 3: Validation of representative genes by qRT-PCR analysis. (A) Expression levels of 13 genes measured on total RNA prepared from SKOV-3 and FT194 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 FT194 cells. (B and C) Expression levels of some representative genes measured on total RNA prepared from FT194 and SKOV-3 cells transiently transfected with PAX8 siRNA or scramble siRNA 24 h (white bars), 48 h (black bars) and 72 h (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 ≤ p ≤ 0.1.
annations co-occurrence discovery web-based tool (GeneCodis; http://genecodis.dacya.ucm.es/). Several significant GO categories appeared enriched including signal transduction, cell adhesion, blood coagulation, positive regulation of cell migration, angiogenesis and cell differentiation (Figure 5A and 5B). At the same time, the most affected pathways upon silencing of PAX8 comprise Wnt signaling, cadherin signaling, integrin signaling and TGF-beta signaling (Figure 5C and 5D).

To further investigate the functional associations of downregulated and upregulated genes following PAX8 knockdown we performed an MsigDB analysis. Interestingly, we observed that for the significantly upregulated genes (87 for SKOV-3 and 47 for FT194) the most affected pathways in both cells lines are interferon response, TNFα signaling, inflammatory response, apoptosis, UV response and epithelial mesenchymal transition (Figure 6). In the same way, for the downregulated genes (214 for SKOV-3 and 119 for FT194) the most affected pathways in both cells lines are epithelial mesenchymal transition, UV response, Kras signaling, estrogen response and p53 pathway (Figure 6). In agreement with our previous studies, the pathways perturbed upon PAX8 silencing such as IFN-γ and TGF-α signaling, EMT, apoptosis, hypoxia as well as UV response strengthen the new role of PAX8 in the regulation of cell survival, proliferation and in the maintenance of oncogenic properties [11, 17].

![Graphs showing binding of PAX8 to target genes](image-url)

**Figure 4: Direct binding of PAX8 on the regulatory regions of putative target genes.** Chromatin immunoprecipitation assays were performed to determine the binding of PAX8 to the 5′-flanking region of representative genes. Chromatin was subjected to quantitative real-time PCR analysis using appropriate primers (see Materials and Methods). Error bars indicate s.d. between two experiments performed in duplicate ($p < 0.001$).
In conclusion, we think that the majority of the genes affected by PAX8 silencing are associated with important biological cellular processes and we believe that our analysis provides a solid basis for the identification of relevant molecules involved in ovarian cancer.

**DISCUSSION**

Recent studies suggest that a substantial proportion of cases of ovarian high-grade serous carcinoma may arise from precursor lesions located in the Fallopian tubal epithelium (FTE). In our study, by means of RNA-seq analysis we investigated the expression of genes modified during the transformation process from Fallopian tube secretory epithelial cells to HGSC cells. Moreover, genes and pathways downstream the transcription factor PAX8 have been analyzed in both cell types. Our goal was to identify new targets for diagnostic and/or therapeutic approaches for HGSC that is the third most common cause of death among gynecologic malignancies worldwide.

Actually, improved screening strategies for HGSC diagnosis in early stages, as well as effective treatments are greatly needed.

We highlighted that ≈ 60% of the genes differentially expressed between the two cell types is modulated at a significant level (FDR-adjusted \( p \)-value ≤ 0.05). Applying a 2 fold-change cutoff and performing a Molecular Signatures Database analysis, we determined in SKOV-3 cells a significant enrichment of estrogen response, EMT, angiogenesis and Wnt/β catenin pathways. At the same time, in FT194 cells we observed an enrichment of pathways like TNF-α, IFN-α, IFN-γ and inflammatory responses. Furthermore, we consider intriguing those genes that turned out to be preferentially, or in some cases exclusively, expressed in FT194 or SKOV-3 cells. There is no doubt that the future challenge will be to examine the potential role of genes differentially expressed between the two cell types as specific and sensitive biomarkers. In fact, several ovarian cancer screening studies have established that acombinatorial biomarker strategy is more

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**Figure 5: Biological processes and pathways altered in siPAX8 cells.** Gene ontology (GO) and Panther pathway analysis have been performed using GeneCodis (http://genecodis.dacya.ucm.es/). (A) GO categories enriched for genes modulated upon PAX8 silencing in SKOV-3 cells. (B) GO categories enriched for genes modulated upon PAX8 silencing in FT194 cells. (C) Panther pathways enriched for genes modulated upon PAX8 silencing in SKOV-3 cells. (D) Panther pathways enriched for genes modulated upon PAX8 silencing in FT194 cells.
reliable, sensitive and specific than using a single protein biomarker [76]. A recent study identified a 5-marker panel for early detection of ovarian cancer that includes five serum biomarkers, namely macrophage-stimulating protein alpha, tissue inhibitor of metalloproteinases-4, platelet-derived growth factor receptor alpha (PDGF-R alpha), osteoprotegerin, and CA-125 [77]. Preliminary insights that we obtained using ProteINSIDE, a novel web service [78], suggest that among the genes highly expressed in SKOV-3 cells there are some that encode for secreted proteins like CGB5 [79], CGB8 [79], FOLR1 [28], SPP1 [80], IGFBP3 [81], NFASC [82] emphasizing that our study may indeed provide a solid basis for the identification of new biomarkers. Of interest, among the top genes highly expressed in SKOV-3 cells there is PRAME that in a wide variety of human malignancies correlates with poor clinical outcome [83]. It has been reported that PRAME proteins are associated to self-renewal cell maintenance [84] and are currently considered as potential target to hamper cancer cell proliferation [85].

In 2003, a gene expression study carried out in ovarian carcinoma [86] reported the transcription factor PAX8, normally absent in ovarian surface epithelial cells, among the most highly expressed genes. The recent carcinogenesis model proposes the secretory cells of the Fallopian tubal mucosa as the cell of origin for the majority of EOC. In agreement with this hypothesis, PAX8 would not be overexpressed in epithelial ovarian carcinoma but rather its expression would be conserved from the cell of origin [38]. To date, the function of PAX8 in Fallopian tube epithelial secretory cells has not been clarified. It has been demonstrated a continually regulated cycle of growth, differentiation, death and renewal in the epithelium of the mammalian oviduct [87] and in this context PAX8 could exert its role. However, more studies are needed to shed light on the function of this transcription factor in this cell type.

We have recently reported that PAX8 is involved in the tumorigenic phenotype of ovarian cancer cells [17]. In the present study, we intend to clarify PAX8 contribution to ovarian cancer through the identification of its downstream gene regulatory network. MsigDB analysis revealed that in both FT194 and SKOV-3 cells the most affected pathways are interferon response, TNFα signaling, inflammatory response, apoptosis, UV response and epithelial mesenchymal transition (considering the upregulated genes) and epithelial mesenchymal transition, UV response, estrogen response and p53 pathway

Figure 6: Pathway analysis of differentially expressed genes after PAX8 silencing. MsigDB software was used to identify the pathways most affected by the gene dysregulation. (A) MsigDB for SKOV-3 downregulated (black bars) and upregulated genes (gray bars). (B) MsigDB for FT194 downregulated (black bars) and upregulated genes (gray bars).
(considering the downregulated genes). Interestingly, our RNA-seq analysis identifies, among PAX8 potential targets, genes that have been reported in the literature having a role in ovarian cancer. One of such genes is FGF18 whose expression is significantly reduced after PAX8 knockdown. It has been demonstrated that FGF18 regulates both tumor cells and tumor microenvironment to facilitate the progression of serous ovarian cancer, enhancing angiogenesis and tumor-associated macrophage infiltration [69, 88]. In addition, FGF18 was identified as the gene possessing the strongest prognostic value in segment 5q31-5q35.3 that was found amplified on microdissected HGSC samples [89]. Furthermore, a recent study provides a platform for the identification of blood-based biomarkers (“Secretome”) for high-grade, advanced-stage serous ovarian tumors and identifies two new markers, FGF18 and GPR172 [90]. Here, we hypothesize that one way in which PAX8 confers a proliferative advantage to ovarian cancer cells is through the regulation of procancer factors, like FGF18, in a pathological microenvironment.

Abnormal activation of the WNT/b-catenin signaling pathway has been associated with ovarian carcinomas. It has been reported that WNT7A is abundantly expressed in ovarian carcinoma and is able to control cell division, adhesion and motility [73]. In addition, WNT7A knockdown cancer cells show a significantly decreased growth rate and invasion ability in a xenografts model [73]. Intriguingly, the phenotype of WNT7A knockdown xenografts resembles that described for the xenograft model of the siPAX8-SKOV-3 cells [17] and this study shows that WNT7A is abundantly expressed only in SKOV-3 cells where is a downstream target of PAX8.

In addition, the expression of some genes regulated by PAX8 in Fallopian tubes secretory cells may be lost during the neoplastic transformation. For example, our data show that chordin (CHRD), a BMP extracellular regulator that behaves as suppressor of tumorigenesis in ovarian carcinoma cells, is abundantly expressed and regulated by PAX8 in FT194 cells, while is almost absent in SKOV-3 cells.

It is worth noting that it has recently been demonstrated that Fallopian tube secretory cell expansion and the ratio between secretory/ciliated cells (S/C ratio) are linked to pelvic serous neoplasia [91] and in this scenario the cancer cells of the serous tubal intraepithelial carcinoma (STIC) bearing a “p53 signature” invade onto the ovary and implant on peritoneal surfaces [92–94]. During this process, the secretory cells retain the expression of PAX8 that possibly continues to exert its transcriptional activity on its physiological targets and in addition may also function on new targets that become available after the tumorigenic hits (BRCA mutations, p53 signature etc).

Our final suggestion is that the relevance of PAX8 in ovarian carcinoma lies in the downstream network(s) regulated by this transcription factor that contribute to ovarian carcinoma pathogenesis. In this respect, we believe that our study has led to the identification of genes and pathways regulated by PAX8 that could be valuable for future studies to uncover the molecular mechanisms leading to EOC.

MATERIALS AND METHODS

Cell culture and RNA interference

The human ovarian carcinoma cell line SKOV-3 was obtained from the CEINGE Cell Culture Facility (Naples, Italy) and was grown in RPMI-1640 medium (Euroclone) containing 10% fetal bovine serum (Euroclone). The immortalized Fallopian tube secretory epithelial cell line FT194 was kindly provided by Dr. R. Drapkin (Boston, USA) and was maintained in DME-F12 medium (Euroclone) containing 2% Ultroser G serum (PALL). The OVCAR-3 cell line was obtained from ATCC and was maintained in RPMI-1640 medium (Euroclone) containing 10% fetal bovine serum (Euroclone) and 0.01 mg/ml bovine insulin. PEA1 and PEO14 cells were purchased from Sigma-Aldrich and maintained in RPMI-1640 medium (Euroclone) containing 10% fetal bovine serum (Euroclone), 2 mM glutamine and 2 mM sodium pyruvate (Gibco, Life Technologies).

For RNA interference, SKOV-3, FT194 cells and PEA1 were plated at 2 × 10^5 cells/60-mm tissue culture dish 24 h prior to transfection and were transfected in replicates with 5 nM PAX8 siRNA (Ambion, Life Technologies, 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 harvested 24 h after transfection and the total RNA was prepared. Human Fallopian tubes RNA was from Origene (CR559726).

RNA extraction, qRT–PCR, RNA-seq and data mining

For both qRT-PCR and RNA-seq experiments, total RNA was extracted using the RNaseasy Mini kit (Qiagen). For qRT-PCR, the cDNA was synthesized using the iScript cDNA Synthesis kit (BIORAD, Hercules, CA). Real time RT–PCR analysis was performed using the IQ™ SYBR Green PCR Master Mix (BIORAD) in a CFX96 Real-Time PCR Detection System (BIORAD) with gene-specific oligonucleotides (Supplementary Table S6).

For RNA-seq, 2 µg of total RNA extracted 24 h after transfection were sent to the Genomics4Life Company (University of Salerno, Italy). Independent silencing experiments were performed from which biological replicates of each condition (siPAX8 or control) were processed for the RNA-seq analysis.

The extracted RNA samples were sequenced using the Illumina HiSeq 1500 platform, TruSeq Stranded Total
RNA, 100 bp paired-end reads at the Genomics4Life Company (University of Salerno, Italy).

Analysis was performed using the EPIGEN project sequence facility, RAP (RNA-Seq Analysis Pipeline) [95], available on the following website https://bioinformatics.cineca.it/. Sequence quality was assessed using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and NGS QC Toolkit [96], using default parameters (PHRED quality score: 20 and for percentage of read length of given quality: 70). Hence, paired-end reads were mapped to the reference human genome build (hg19/GRCh37) using Tophat 2.0.12 [97] with default parameters. The resulting alignment files are provided to Cufflinks [98] to generate a transcriptome assembly and to estimate the expression level expressed as units of FPKM (Fragment mapped per kilobase of exons per million mapped reads). Differential expression analyses were performed with Cuffdiff2 [99] using default parameters. An alpha level of 0.05 was used for all statistical tests. Gene expression data have been submitted to the Gene Expression Omnibus (GEO), Accession number GSE79572.

Chromatin immunoprecipitation assay

ChIP was performed as previously described [100]. Precleared chromatin from FT194 cells was incubated with 3 µg of affinity-purified rabbit polyclonal anti-PAX8 antibody (Thermo Scientific, PA1-112) or polyclonal anti-TAZ antibody as unrelated (Santa Cruz Biotechnology, sc-17130) and rotated at 4°C for 16 h. Thereafter, the immunoprecipitated DNAs were amplified by quantitative real-time PCR with the following primers:

- FGF18 for. 5′-gtgggtagccagtcaagagg-3′; rev. 5′-ctccccaagaacgcagttag-3′;
- ANXA2 for. 5′-Gctaaacggctgcaagaaac-3′; rev. 5′-Cgtagcaggcagtcctgag-3′;
- CDH6 for. 5′-Atccaacagtggctgactcc-3′; rev. 5′-tctggaaagttgccgaagtt-3′;
- ROR1 for. 5′-CAGATCACAGCTGCCTTCAC-3′; rev. 5′-ATTTCACATTCATCGCGACA-3′.

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 [101–103].

Genes showing a two-fold change in expression were analyzed at Molecular Signatures Database (http://software.broadinstitute.org/gsea/msigdb) using the “Hallmark” gene set.

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CONFLICT OF INTEREST

The authors declare that they have no known conflicts of interest in this work.

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