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FOXO3a loss is a frequent early event in high-grade pelvic serous carcinogenesis

Keren Levanon[1,2,†,*], **Stav Sapoznik**[3,*], **Keren Bahar-Shany**[3], **Hadar Brand**[3,4], **Ronnie Shapira-Frommer**[4], **Jacob Korach**[6], **Michelle S. Hirsch**[7], **Michael H. Roh**[7], **Alexander Miron**[8], **Joyce F. Liu**[8,9], **Natalie Vena**[10], **Azra H. Ligon**[6,9,10], **Susan Fotheringham**[10], **Dyane Bailey**[10], **Richard J. Flavin**[10], **Michael J. Birrer**[11], and **Ronny I. Drapkin**[2,7,9]

[1]The Dr. Pinchas Borenstein Talpiot Medical Leadership Program 2012, Sheba Cancer Research Center, Chaim Sheba Medical Center; Ramat Gan, 52621; Israel

[2]Department of Medical Oncology; Dana-Farber Cancer Institute; Boston, MA, 02115; USA

[3]Sheba Cancer Research Center; Chaim Sheba Medical Center; Ramat Gan, 52621; Israel

[4]Institute of Oncology, Chaim Sheba Medical Center; Ramat Gan, 52621; Israel

[5]Sackler Faculty of Medicine; Tel-Aviv University; Ramat Aviv, 69978, Israel

[6]Department of Gynecologic Oncology; Chaim Sheba Medical Center; Ramat Gan, 52621; Israel

[7]Department of Pathology; Brigham and Women's Hospital; Boston, MA, 02115; USA

[8]Department of Cancer Biology; Dana-Farber Cancer Institute; Boston, MA, 02115; USA

[9]Harvard Medical School; Boston, MA, 02115; USA

[10]Center of Molecular Oncologic Pathology; Dana- Farber Cancer Institute; Boston, MA, 02115; USA

[11]Center for Cancer Research; Massachusetts General Hospital; Boston, MA, 02114; USA

Abstract

Serous ovarian carcinoma is the most lethal gynecological malignancy in Western countries. The molecular events that underlie the development of the disease have been elusive for many years. The recent identification of the fallopian tube secretory epithelial cells (FTSECs) as the cell-of-origin for most cases of this disease has led to studies aimed at elucidating new candidate therapeutic pathways through profiling of normal FTSECs and serous carcinomas. Here, we describe the results of transcriptional profiles that identify the loss of the tumor suppressive transcription factor FOXO3a in a vast majority of high grade serous ovarian carcinomas (HGSOCs). We show that FOXO3a loss is a hallmark of the earliest stages of serous carcinogenesis and occurs both at the DNA, RNA and protein levels. We describe several

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[†]Corresponding Author: Keren Levanon, MD, PhD, Sheba Cancer Research Center, Sheba Medical Center, Ramat Gan 52621, Israel, Tel: (+972)3-5308431 Fax: (+972)3-5305942, Keren.Levanon@sheba.health.gov.il.
[*]These authors contributed equally to this work.

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mechanisms responsible for FOXO3a inactivity, including chromosomal deletion (chromosome 6q21), upregulation of *miRNA-182* and destabilization by activated PI3K and MEK. The identification of pathways involved in the pathogenesis of ovarian cancer can advance the management of this disease from being dependant on surgery and cytotoxic chemotherapy alone to the era of targeted therapy. Our data strongly suggest FOXO3a as a possible target for clinical intervention.

Keywords

Serous ovarian carcinoma; fallopian tube epithelium; FOXO3a; AKT; ERK; miRNA-182

Introduction

Despite a decade of emerging targeted therapies in oncology, the standard of care for ovarian carcinoma remains dependent on cytotoxic drugs (1). An unsurpassed obstacle in the effort to better understand this disease has been the fact that the benign tissue-of-origin has been difficult to identify until recently. For decades the ovarian surface epithelium (OSE), has been viewed as the tissue-of-origin of all types of ovarian carcinoma (2). However, with respect to high-grade serous ovarian carcinomas (HGSOC), the most abundant and most lethal histological subtype, this hypothesis has been challenged by two facts: (1) serous carcinoma displays features of Müllerian epithelium, which is more highly differentiated than OSE; and (2) precursor and early lesions in direct continuity with the advanced high-grade carcinoma has never been reliably demonstrated in the OSE. Recent work revealed the existence of early neoplastic lesions (serous tubal intraepithelial carcinoma, STIC) in the distal fallopian tube (FT) fimbria in the majority of both hereditary and sporadic cases of HGSOC (3, 4). These STICs share identical mutations in the tumor suppressor gene *TP53* with the adjacent invasive carcinoma, suggesting causal relations (3). Additionally, precursor lesions, termed ‘p53 signature’, which also harbor aberrations in *TP53* but are morphologically benign and non-proliferative, can also be detected in the fimbria (5), suggesting that the distal fallopian tube fimbria is a putative field-of-origin for some serous ovarian carcinoma. Previous studies compared the transcriptome, proteome, and epigenome of serous ovarian carcinoma with that of the OSE. However, with the emergence of the FT epithelium (FTE) as a putative field-of-origin it is necessary to define the genomic aberrations that define the oncogenic process transforming FT secretory cells (FTSECs) into serous carcinoma. Such studies have the potential of discovering more promising therapeutic approaches.

In this work we took a genome-wide approach and compared the expression profiles of pure FTE cells, immortalized FTSECs and a collection of micro-dissected HGSOCs. While this comparison highlighted several targets that have already been discovered in studies that used OSE as a normal counterpart, it has also prompted the discovery of new candidate genes and pathways. One such player is FOXO3a, a forkhead family transcription factor that functions as a tumor suppressor (6, 7). FOXO3a has been previously shown to be a pivotal controller of apoptosis and cell cycle (8), glucose metabolism (9), and longevity in invertebrates (10). It is regulated by growth factors signaling pathways, through phosphorylation by activated

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AKT or activated ERK, shuttling to the cytoplasm and proteosomal degradation (11, 12). Conversely, deprivation of growth factors (13), metabolic stress, and increased radical oxygen species (ROS) production (14), cause nuclear retention of FOXO3a and enhancement of its transcriptional activity mostly via phosphorylation on different sites by AMP-activated protein kinase (AMPK) (9). Targets of FOXO3a include Bim (8), CDKN1B ($p27^{kip1}$), (15), FOXM1 (16)Fas ligand (CD95L), cyclin D1, MnSOD, catalase, and GADD45a (17).

The contribution of FOXO3a to ovarian cancer was previously described. Fei et al. reported lower expression of FOXO3a in malignant ovarian tumors than in normal tissues. Negative tumors were significantly associated with short overall survival (18). Lu et al. showed that FOXO3a expression correlated significantly with disease stage and lymph node involvement (19).

A role for FOXO3a in cancer progression has been described also for breast cancer(20), colon cancer (21), lung cancer (22, 23), AML (24), glioma (25) and neuroblastoma (26). Being a tumor suppressor which regulates cell cycle arrest and apoptosis (27), loss of FOXO3a was shown to be positively correlated with disease aggressiveness. Consequently, down regulation of the protein activity resulted in poor prognosis and reduced overall survival (18, 19, 24). Interestingly, beta-catenin was found to confer resistance to FOXO3a-mediated apoptosis in colon cancer. In the presence of high nuclear beta-catenin content, activation of FOXO3a induced tumor metastasis rather than tumor suppression (21).

Several studies have shown that FOXO3a is an important determinant of response to chemotherapy and targeted therapy (28). The cytostatic and cytotoxic effects of various chemotherapies including cisplatin (23), and paclitaxel (29), are mediated by FOXO activation (30). Similarly, transcriptional FOXO3a activity is required for radiosensitization (22).

Though the involvement of FOXO3a in ovarian cancer was previously described (18, 19), the exact stage and mechanisms of its downregulation are not fully understood. In the current study we show that FOXO3a transcriptional activity is lost both in early and late stages of high-grade serous carcinoma. We describe various means by which this important tumor suppressor is inactivated at the genomic, transcriptional and functional levels. We suggest that targeted therapy which restores FOXO3a function may have favorable impact on the prognosis of this disease and we therefore propose the FOXO3a pathway as a possible target for clinical intervention.

Results

Comparison of normal FT epithelium to high-grade pelvic serous carcinomas

We compared HGSOCs with their benign tissue-of-origin, the fallopian tube epithelium (FTE), and particularly the presumed cell-of-origin: the fallopian tube epithelial secretory cells (FTSEC). FTE was harvested from fresh FT fimbria specimens removed from patients undergoing gynecological surgery for non-malignant indications. We overexpressed the human telomerase catalytic domain (*hTERT*) in the primary FTE cells to create

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immortalized cell lines. Usage of retroviral vectors for the infection provided selectivity to proliferative cells only, i.e. FTSECs. These cells maintain expression of epithelial markers such as EpCAM and cytokeratin 7, as well as specific lineage markers, like Pax8, and can be propagated for over 15 passages with no detectable change in morphology (31, 32).

We used GeneChip© Human Genome U133 Plus 2.0 array (Affymetrix) to profile 3 of the human telomerase (*hTERT)*-immortalized FTSEC lines that were developed in our laboratory, as well as mixed primary epithelial cells isolated from 10 fresh benign FT fimbria, and 60 micro-dissected late stage HGSOC samples. Using cutoff values of *p*-value <0.001 and fold change >1.5, the number of genes that were up-regulated in serous tumors was 2220 and 1352 compared to mixed FTE and immortalized FTSECs, respectively, with 882 genes in common ($p=5\times10^{-50}$). Additionally, 2007 and 1629 genes were down-regulated in tumors compared to mixed FTE and immortalized FTSECs, respectively, with 950 genes in common ($p=4\times10^{-11}$).

FOXO3a is decreased in vast majority of HGSOCs

Among these differentially expressed genes we found *FOXO3a* to be decreased 2.9-fold in tumors compared to FTE (p=2.1E-6). Figure 1a shows a heat-map representing the expression levels of FOXO3a and its known target genes in normal FTE samples, compared to HGSOC samples. We validated this finding by real-time PCR and found a 7.5 fold reduction in FOXO3a mRNA in late-stage HGSOC compared to normal FTE specimens (Figure 1b, $p=2.98\times10^{-5}$, n=12 and n=11 respectively). Next, in order to test the change in protein level of FOXO3a, we stained a tissue microarray (TMA) containing representative cores from 134 formalin-fixed paraffin embedded (FFPE) late-stage (International Federation of Gynecology and Obstetrics (FIGO) stage III–IV) HGSOC cases. 115 out of 128 (90%) tumors that qualified for scoring scored either 0 (no staining, 77/128, 60%) or 1 (weak or focal staining, 38/128, 29.6%) while 13 out of 128 (10%) tumors scored either 2 or 3 (moderate or strong staining, Figure 1c, Supplementary Figure 1a–c). Staining of normal FT fimbria showed predominantly nuclear localization of FOXO3a (Supplementary Figure 1d). In view of the recent identification of the precursor lesions (‘p53 signatures’) and early serous tubal intraepithelial carcinoma lesions (STICs) in the FTE, we wanted to define the stage in the oncogenic spectrum at which the decrease in the levels of FOXO3a protein becomes detectable. We stained FFPE sections of FT fimbria containing both normal epithelium, ‘p53 signature’, STIC and an invasive serous tumor from 10 different patients, for FOXO3a. The histological stage in the spectrum of the carcinogenic transformation was defined using Hematoxylin and Eosin (H&E), TP53 immunostain, and Ki67 immunostain, serving as a proliferation marker. We saw that the level of FOXO3a in ‘p53 signatures’ was similar to that seen in the normal FTE, but decreased dramatically in the transition to neoplasia (Figure 2). In some cases FOXO3a was lost at the STIC stage and in some cases only at the progression to an invasive tumor. Likewise, the level of CDKN1B/$p27^{kip1}$, a target of FOXO3a, decreased in the exact corresponding stage (15). Conversely, FOXM1, which is negatively regulated by FOXO3a, displayed inverse correlation and was increased in the STICs and/or invasive tumors compared to the earlier steps of oncogenesis (16). Put together, these data suggest that the down-regulation of FOXO3a in serous carcinoma occurs both at the mRNA and at the protein levels. This phenomenon is not only restricted to late-

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stage disease as represented by our TMA, but can already be tracked to the transition from a precursor lesion to an intraepithelial carcinoma in the FT, several steps before it becomes disseminated.

Several mechanisms contribute to the loss of FOXO3a mRNA in HGSOC

Our next goal was to define the mechanism responsible for the decrease in FOXO3a expression in HGSOC. First, we tested for allelic loss of FOXO3a, which maps to 6q21, using fluorescence in situ hybridization (FISH) using BAC probe RP11-1150O2 (CHORI(33)) which encompasses the full gene length, and the CEP6 probe (Abbott Molecular) that hybridizes to 6p11.1-q11. We performed FISH on late-stage HGSOC tumors that did not express FOXO3a by IHC, and scored 100 cells for each tumor sample as either: (1) disomic for FOXO3a (two CEP6 signals and two FOXO3a signals per nucleus), (2) polysomic (equal number of CEP6 and FOXO3a signals, with greater than two copies of each probe), (3) deletion (or relative deletion) involving FOXO3a, or (4) nullisomy for FOXO3a (no FOXO3a signals, Supplementary Figure 2a–f). We found that six out of nine tested tumors had FOXO3a deletion in more than 30% of the cells (Supplementary Figure 2g). Our results are in agreement with array comparative genomic hybridization (aCGH) performed on 559 HGSOC tumor specimens by The Cancer Genome Atlas project showing very high rate of heterozygous loss of FOXO3a (299/559 tumors, 52%) and homozygous loss in 1.4% (8/559 tumors). The analysis was performed using the cBioPortal (http://www.cbioportal.org/public-portal) (33).

Another potential mechanism of transcriptional down-regulation is promoter methylation. A large CpG island that contains 432 CG dinucleotides extending from the FOXO3a promoter into the second exon of the gene, and which is not normally methylated has been defined using the University of California Santz Cruz (UCSC) Genome Browser (http://genome.ucsc.edu/(34)). CpG islands that undergo *de novo* methylation in cancer tend to involve local trimethylation of histone H3 on lysine 27 (H3K27me3)(35) and Polycomb components (36). The promoter region of FOXO3a is not enriched for H3K27me3 and other Polycomb elements (UCSC Genome Browser (37)), making it less likely to undergo *de novo* methylation. In order to validate this prediction, we tested a region spanning 292 bp upstream of the translation start site in 10 tumors that did not express FOXO3a by IHC. The DNA underwent bisulfite conversion of non-methylated cytidines into thymidines (EpiTECT, Qiagen) and the specific region was amplified using 2 sets of primers that were complementary with either bisulfite-converted or genomic DNA sequences. The amplification products were cloned and sequenced. We found no evidence of hypermethylation in this promoter region. We thus conclude that *de novo* methylation of the FOXO3a promoter is unlikely to account for the widespread down-regulation of FOXO3a in HGSOC.

Next we wanted to test the possibility that FOXO3a is silenced by a specific micro-RNA (miRNA). We searched for putative miRNAs that target the 3′ UTR of the FOXO3a gene using the TargetScan miRNA prediction tools provided by the UCSC Genome Browser. The only predicted miRNA with an evolutionary highly conserved 8-nucleotide-long (8mer) seed within the FOXO3a 3′UTR, was *miRNA-182* which was also reported to target FOXO3a

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mRNA in melanoma cells (38). We performed quantitative RT-PCR and found *miRNA-182* to be significantly upregulated in HGSOCs compared to normal FTE (Figure 3a, p=0.008, n=6, n=5 respectively). We further validated this finding by *in-situ* hybridization (ISH) with an anti-sense *miRNA-182* probe (Exiqon) and found that *miRNA-182* is mildly up-regulated in HGSOC tumors compared to normal FT epithelium, with scrambled miRNA serving as a normalizing control (Figure 3b). In order to validate the effect of the observed elevation in *miRNA-182* on FOXO3a expression, we transfected either *miRNA-182* or a control miRNA into a commercial cell line of ovarian carcinoma (HeyA8). Notable reduction in FOXO3a protein levels, as well as in its downstream target CDKN1B/p27^{kip1}, was detected by immunoblotting (Figure 3c). We therefore conclude that *miRNA-182* has a role in the down-regulation of FOXO3a mRNA and protein levels making it a compelling target for further investigation and potential therapeutic intervention. It is noteworthy that in the provisional ovarian serous carcinoma TCGA dataset *miR-182* is amplified in 53 (9%) out of 559 cases and up-regulated in additional 20 (4%) out of 526 cases.

Finally, we searched for inactivating mutations in the FOXO3a coding sequence. FOXO3a has a non-coding pseudogene, i.e FOXO3b, on chromosome 17 (chr17:18,509,961–18,517,219), with 98.5% sequence identity. We performed partial sequencing using specific primer pairs that distinguish the two variants on genomic DNA extracted from 10 FFPE specimens of HGSOCs that do not express FOXO3a by IHC, and from 10 random samples of tumor cells harvested from ascites of advanced HGSOC patients. No mutations within the coding sequence where detected. Our data is compatible with the TCGA database which report mutations of unknown significance in only 2 (0.6%) out of 316 sequenced tumor samples.

The AKT and ERK pathways are involved in FOXO3a inactivation in ovarian tumors

The transcriptional function of FOXO family members, including FOXO3a, is known to be regulated by phosphorylation and ubiquitin-proteasome-mediated degradation (11). FOXO3a has been reported to be phosphorylated by activated AKT and activated MEK in response to growth stimuli and is consequently bound by 14-3-3 and exported from the nucleus to be degraded by the proteasome. However, when trying to decipher the mechanisms underlying the widespread down-regulation of FOXO3a in HGSOC, it is important to keep in mind that AKT- or ERK-mediated degradation can only provide explanation for the decrease in FOXO3a protein levels and not for the decrease at the mRNA levels, as seen by us. In order to determine the clinical relevance of these two pathways in HGSOC, we performed immunohistochemical (IHC) staining for phospho-AKT (pS473), stathmin (encoded by the gene *STMN1*, previously reported as an accurate IHC marker for aberrant phosphatidylinositol-3-kinase (PI3K) signaling (39)), and phospho-ERK1/2 (pT202/pY204) on the different stages in the spectrum of serous carcinogenesis. Whereas no change in phospho-AKT was observed, enhanced activation of the PI3K pathway, as determined by expression of stathmin, was identified as early as in the ‘p53 signature’ stage, and in the more advanced intraepithelial and invasive carcinoma (Figure 4). The RAS/MEK pathway was activated in the ‘p53 signature’ stage, as determined by IHC staining for phosphorylated ERK (p42/p44-MAPK) (Figure 5). Interestingly, MEK pathway activation occurs as early as in the ‘p53 signature’ stage, but FOXO3a loss requires an

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additional genetic event that coincides with the progression from ‘p53 signature’ to STIC, which correlates with the rarely seen entity termed ‘p53 signature in transition’, characterized by benign morphology and an increase in the proliferation index Ki67 (Figure 5) (40).

Once we established this, we wanted to test the role of the PI3K/AKT and the RAS/MEK/ERK pathways in the regulation of FOXO3a cellular localization and activity in HGSOC cells. To do that we pharmacologically inhibited these pathways in a panel of established cell lines and primary tumor cells derived from patients’ ascites fluid. Inhibition of the PI3K pathway was achieved by adding 20 μM LY294002 to the cell media for 24 hours, while inhibition of the MEK pathway was achieved by adding 10 μM U0126 to the cell media for 24 hours. In most ovarian cancer cell lines tested, as well as in primary tumor cells derived from ascites fluid of patients, inhibition of the PI3K-AKT pathway resulted in accumulation of FOXO3a protein in the nucleus and a subsequent expression of CDKN1B/$p27^{kip1}$ (Figure 6a–c). Similar effect was seen when the RAS/MEK/ERK pathway was inhibited by U0126. The combination of LY294002 and U0126 did not result in synergistic effect (Figure 6a–c).

The ability to restore FOXO3a nuclear activity by pharmacological means may have therapeutic implications. We therefore examined the effect of the PI3K and the MEK inhibitors on cell viability. Using LY294002 and U0126 we could indeed detect a reduction in cell viability as detected by the CellTiter Glo assay (Promega, Figure 6d).

Overall, our findings suggest a major role for activated PI3K/AKT and RAS/MEK/ERK pathways in nuclear exclusion of FOXO3a and inhibition of its tumor suppressive function in many ovarian cancer cell lines and primary tumors. The activation of these pathways is clinically significant and occurs at the earliest stages of transition from normal FTE to a ‘p53 signature’. Drugs that inhibit these pathways cause nuclear translocation of FOXO3a leading to restoration of FOXO3a-mediated signaling.

Discussion

The concept of the fallopian tube fimbria being the tissue-of-origin of most pelvic serous carcinoma and the FTSEC being its cell-of-origin, is translated in this manuscript into the first rigorous comparison of the normal FTE transcriptome to that of HGSOCs. Among many candidates that await exploration we focused on FOXO3a which is already known as a tumor suppressive transcription factor. The loss of FOXO3a has been reported before (18), but the extent of this phenomenon has never been studied. Epithelial tumors are often characterized by a multitude of genetic, epigenetic and post-transcriptional events, including inactivating mutations, rearrangements, copy-number variations, hypermethylation, and destabilization of mRNA by miRNA, each of them occurring in a limited subset of tumors within a single pathological classification. We hereby report that FOXO3a is significantly suppressed both at the mRNA and at the protein levels in up to 90% of HGSOCs. One possible approach to distinguish between causative aberrations (‘driver events’) and secondary aberrations in tumor cells (‘passenger events’) is by looking at the mechanisms leading to the common end result. Intuitively, we assume that various aberrations are

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introduced into cells in a random fashion, but only those that convey a positive selection advantage to the pre-neoplastic or neoplastic cell survive and can be detected in the fully developed tumor. Therefore, if a specific abnormality is at the convergence point of various mechanisms, it is more likely to be of greater selection benefit to the cells. Along these lines we report that several mechanisms, including chromosomal deletions, miRNA overexpression and increased phosphorylation, all contribute to the net effect of FOXO3a suppression.

Activation of the PI3K and RAS/MEK pathway occur at the earliest stage of serous carcinogenesis known so far – at the 'p53 signature' stage. At this stage *TP53* has already been mutated in many of the cases, or has become dysfunctional, though only a small minority of these lesions will evolve into cancer (41). The complete loss of FOXO3a is witnessed only at the STIC stage or at the invasive fallopian tube carcinoma, and in rare occasions at an intermediate lesion between 'p53 signature' and STIC termed 'p53 signature in transition' (40). It is possible that another event, directly involving the FOXO3a locus, either an inactivating mutation or deletion, is needed to allow the 'p53 signature' to breach the cell cycle checkpoints and progress into a proliferative lesion.

Our data further highlights the significance of the PI3K and RAS/MEK pathways in HGSOC. We show that by inhibiting these pathways in cell lines, FOXO3a cellular localization and transcriptional activity is restored, and its target gene CDKN1B/$p27^{kip1}$ is expressed. This data suggests that clinical trials with PI3K and MEK inhibitors, may be appropriate for HGSOC patients, and that FOXO3a function can serve as a read-out for on-target effect of the drugs. However, it is noteworthy that we detected FOXO3a expression in cell lines cytoplasm and in primary tumor cells cytoplasm when grown *in vitro* (Supplementary figure 3), while FOXO3a was not expressed by most tumors in-vivo. We observed differences between cultured primary cells and FFPE tumors from the same patient in several cases, implying to the existence of a yet unknown factor in the culture medium or culture conditions that restores some FOXO3a expression, though it is still non-functional as it is located to the cytoplasm of the cell only. We thus suspect that a patient-tailored approach of testing for response to PI3K or MEK inhibition on cultured primary tumor cells, may not give an absolutely reliable indication about the potential of this drug in the clinical setting.

Previous literature reports only modest neoplastic phenotype of animals in which FOXO genes have been knocked-out (42). FOXO3a $^{-/-}$ are not particularly prone to develop cancer, however females suffer from premature ovarian failure (43). Some explanations for this observation include the hypotheses that FOXO3a function alters the tumor microenvironment: it is an inhibitor of angiogenesis (44), and a modulator of the immune response (45). The exact mechanisms through which FOXO3a loss is advantageous for cancer cells is yet to be discovered, but it provides an opportunity for therapeutic targeting of the FOXO3a signaling pathway via its down-stream targets, as an alternative to attempting to directly restore its function.

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Experimental Procedures

FTSEC immortalized cell lines

Fresh FT fimbria specimens were obtained with approval of the institutional review board. The fimbria tissues were incubated in a dissociation medium (EMEM (Cellgro) supplemented with 3.5 mg/mL Pronase (Roche Diagnostics) and 0.25 mg/mL DNase (SIGMA)), for 48–72 hrs in 4°C with constant mild agitation. The dissociated epithelial cells were harvested by centrifugation and re-suspended in DMEM/ Ham's F12 1:1 (Cellgro), supplemented with 2% serum substitute Ultroser G (Pall France) and 1% penicillin/ streptomycin (PS, Invitrogen). Cells were plated on culture-ware that was treated with human collagen IV (SIGMA, 0.06 mg/mL in water) overnight, and incubated at 37°C in a humidified 5% CO_2 incubator. Immortalization was performed using the human telomerase (*hTERT*)-expressing amphotropic retroviral vectors (46).

Primary tumor cells

With institutional review board approval, primary ovarian carcinoma cells were isolated directly from peritoneal paracentesis' from patients with advanced stage ovarian cancer at the time of initial cytoreductive surgery or subsequent to treatment. Red blood cells were lysed using hypotonic NaCl solution. tumor cells were cultured in RPMI 1640 medium (Cellgro) supplemented with 10% fetal bovine serum (FBS, Gibco).

Ovarian cancer cell lines and drug intervention

The following ovarian carcinoma cell lines were grown in RPMI 1640 medium supplemented with 10% FBS and 1% PS: OVCAR8, SKOV3, OVCA420, OVCA429. IGROV1 cells were grown in DMEM medium containing 10% FBS and 1% PS. OVCAR3 cells were grown in MCDB:199 (1:1) medium containing 15% FBS and 1% PS. All cell lines were bought from the ATCC cell bank. Growth medium for FTSEC lines was DMEM/ Ham's F12 1:1, supplemented with 2% serum substitute Ultroser G (Pall France) and 1% PS. All the cells are incubated at 37°C in a humidified 5% CO_2 incubator. LY294002 (SIGMA) and U0126 were reconstituted in DMSO and used at final concentration of 20μM and 10 μM respectively. Cells were exposed to the various drugs for 24 hours.

Cell viability assay

The effect of drug treatment on cell viability was studied using the CellTiter Glo assay (Promega).

Expression profiling

RNA from cells was extracted using TRIZOL reagent (Invitrogen) followed by RNeasy clean-up kit (Qiagen). Expression profiling was performed using GeneChip© Human Genome U133 Plus 2.0 array (Affymetrix). Statistical analysis of the results was done using Fisher's Exact Test.

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qRT-PCR

qRT-PCR was performed on the 7900HT Fast Real Time PCR System (Applied Biosystems). FOXO3a mRNA levels were examined using FastSYBR Green MasterMix (Applied Biosystems), and the following primer sequences: FOXO3a: 5′ CCCAACCAGCTCCTTTAACA, 5′ GAGTCCGAAGTGAGCAGGTC, GAPDH: 5′ CCTGTTCGACAGTCAGCCG, 5′ CGACCAAATCCGTTGACTCC. miRNA-182 levels were examined using SYBR Green master mix (Exiqon) with has-miR-182-5p and has-miR-103 primers (Exiqon). Data are presented as mean value ± standard error. Statistical significance was assessed by t test.

Tissue microarray

HGSOC were collected under the approval of the Institutional Review Board from patients undergoing primary cytoreductive surgery. Four cores of tissue (0.8mm in diameter) were taken from each case.

Immunoblotting, immunohistochemistry, and immunofluorescence

Staining and immunoblotting were carried out using anti p53 (Immunotech), anti Ki67 (Abcam), anti FOXO3a (Cell Signaling), anti p27 (BD Transduction Laboratories), anti FOXM1 (Santa Cruz, positive control: ovarian carcinoma), anti pAKT (Cell Signaling, positive control: breast carcinoma), anti Stathmin (Cell Signaling, positive control: prostate carcinoma) and anti pERK (Cell Signaling, positive control: breast carcinoma) antibodies. Secondary antibodies were purchased from Jackson ImmunoLabs, from Vector Laboratories, and from Amersham Laboratories. To characterize the FOXO3a antibody for IHC it was first tested by Western blot and immunofluorescence in culture cells transfected to over-express FOXO3a. Specificity was also inferred by the differential positivity between different tissue compartments (epithelial versus stroma vs endothelium). Non-immune rabbit IgG antibodies and omission of the primary antibody were used as negative controls.

Fluorescent in-situ hybridization (FISH)

FISH was performed on 5 micron FFPE tissues, which were baked at 60°C for at least two hours, de-paraffinized and digested (47). BAC probe RP11-115O02 was obtained from the CHORI BACPAC Resources Center, Oakland, CA, USA, and used at 100ng/ul.. The CEP6 probe was obtained from Abbott Molecular, Inc., IL, USA, and used according to manufacturer’s directions. Tissue sections and probes were co-denatured, hybridized at least 16 hrs at 37°C in a darkened humid chamber, washed in 2X SSC at 70°C for 10 min, rinsed in room temperature 2X SSC, and counterstained with DAPI (Abbott Molecular/Vysis, Inc.). Slides were imaged using an Olympus BX51 fluorescence microscope.

DNA Methylation detection

DNA was extracted from FFPE tumors and digested by Hind III. Bisulfite reaction was performed using the EpiTECT Bisulfite kit (Qiagen). The desired promoter region was amplified using two sets of primers, matching the genomic sequence and the putative bisulfite converted sequence in the same region, cloned into pGEM-T vectors (Promega): 5′ CACTTCCAAAAAATAAAAAAAAA, 5′ GTTAGGTTAGGAAAGGGGAGAAG, 5′

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CTCCCAAAAACAAAAAAACCTAA. Bacterial clones were subjected to sequencing using standard T7 and Sp6 primers (Genewiz).

in-situ hybridization

In situ hybridization was carried out using the miRCURY LNA™ microRNA ISH Optimization Kit (FFPE) with specific DIG labeled has-miR-182* and scramble-miR probes (Exiqon).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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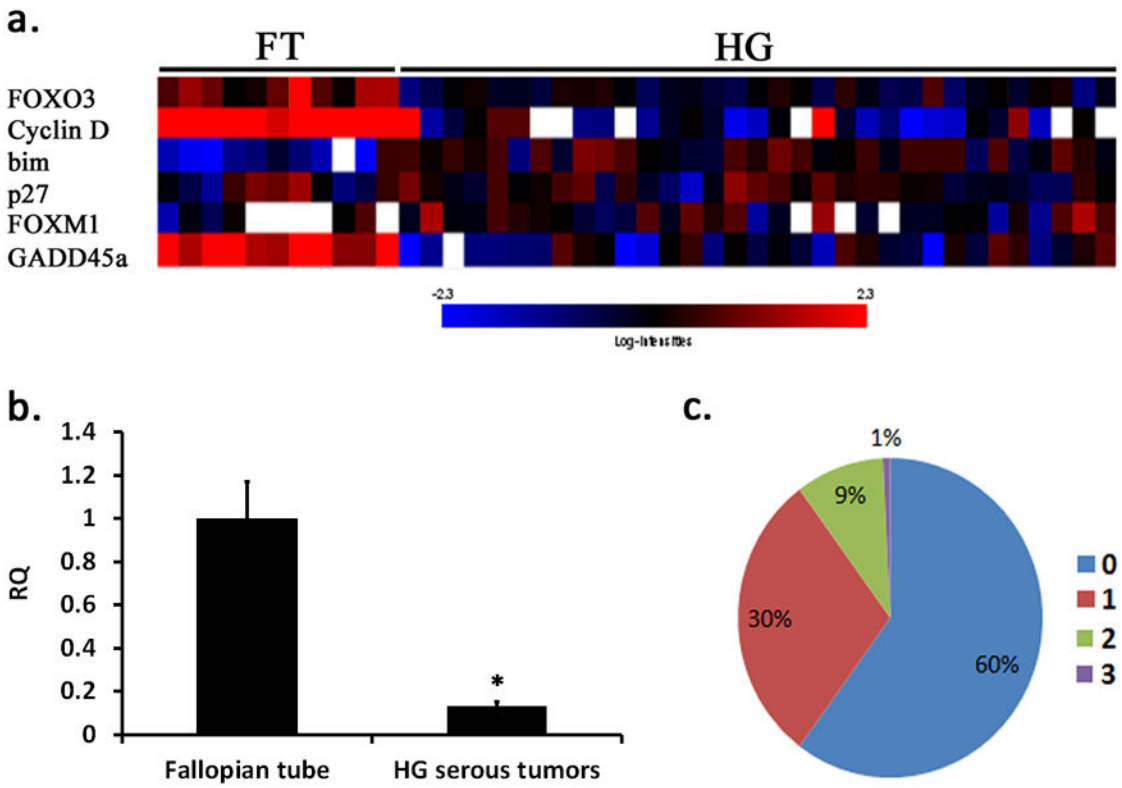

Figure 1. FOXO3a is down-regulated in late-stage HGSOC tumors

a, Heat-map showing decrease in FOXO3a and several target genes, in HGSOC compared to normal FTE. **b,** qRT-PCR using FOXO3a primers on late-stage HGSOC compared to normal FTE, using a different set of cases. **c,** Percentage of tumors in the HGSOC TMA in each staining intensity score category '0' (no immune-staining for FOXO3a), '1' (weak or focal staining), '2' (moderate staining), '3' (strong staining).

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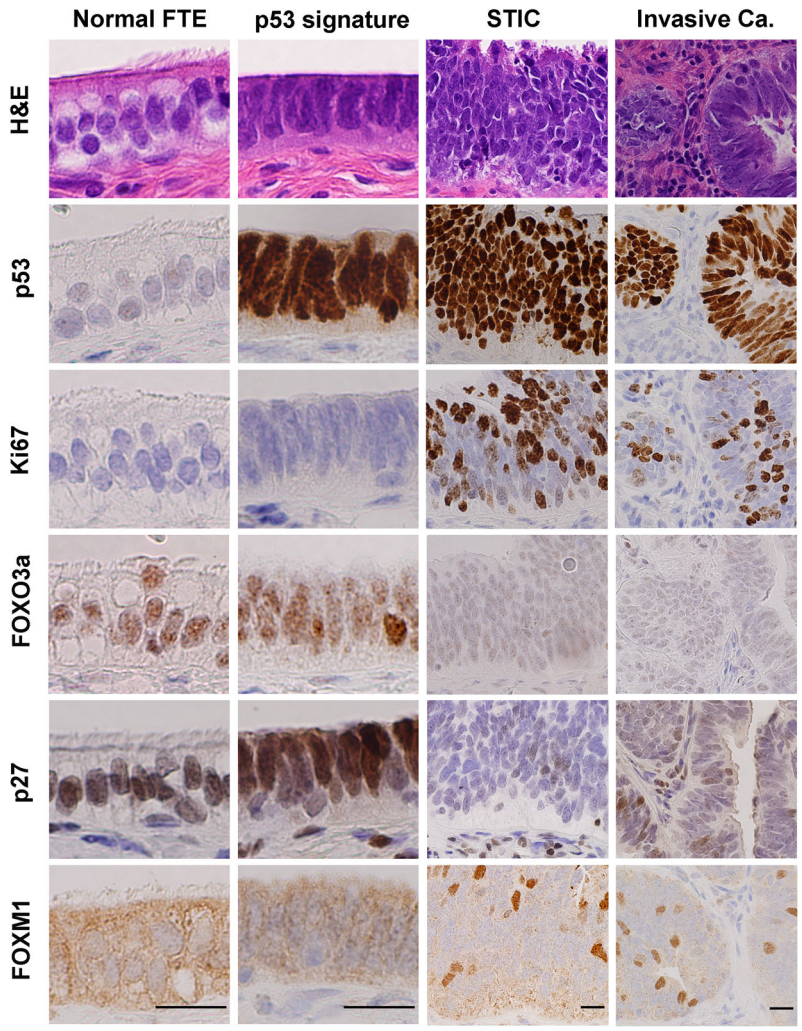

Figure 2. FOXO3a is down regulated in early stages of serous carcinogenesis

H&E and immuno-staining of normal FTE, 'p53 signature', STIC and invasive serous carcinoma from a single patient indicating that FOXO3a is lost in the STIC phase. Staining for p53 indicates the boundaries of the precancerous and cancerous lesions, Ki67 staining distinguishes between carcinoma and 'p53 signature' (negative for ki67). FOXO3a is present at the normal and 'p53 stages' but absent at the STIC and invasive carcinoma stages. Staining for FOXO3a targets $p27^{kip1}$ and FOXM1, shows corresponding down-regulation of $p27^{kip1}$ in STICs and invasive carcinoma, while FOXM1 inversely correlates with FOXO3a expression and is up-regulated in HGSOC tumors. Bar - 20 μm

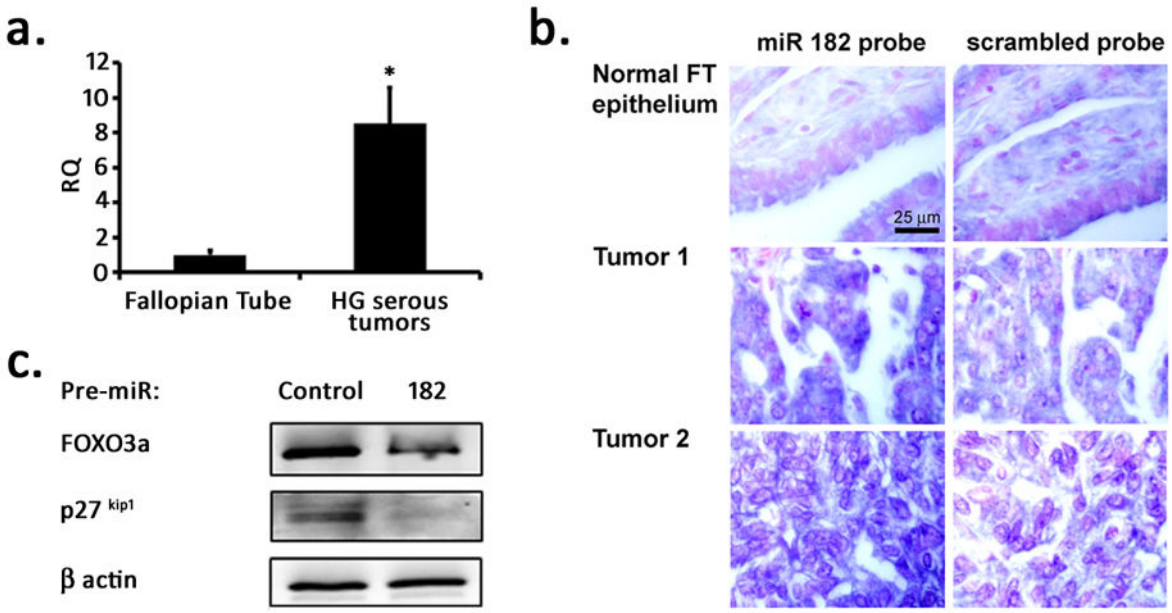

Figure 3. Mechanisms underlying the down regulation of FOXO3a

a, qRT-PCR demonstrating over-expression of miRNA-182 in HGSOC, compared to normal FTE. **b,** In situ hybridization of miR182 or a scrambled probe on tumor and normal FT epithelium. Bar-25 μm. **c,** Western blot analysis of FOXO3a and p27Kip1 in Aey8 cells transfected with miR182 or a control plasmid.

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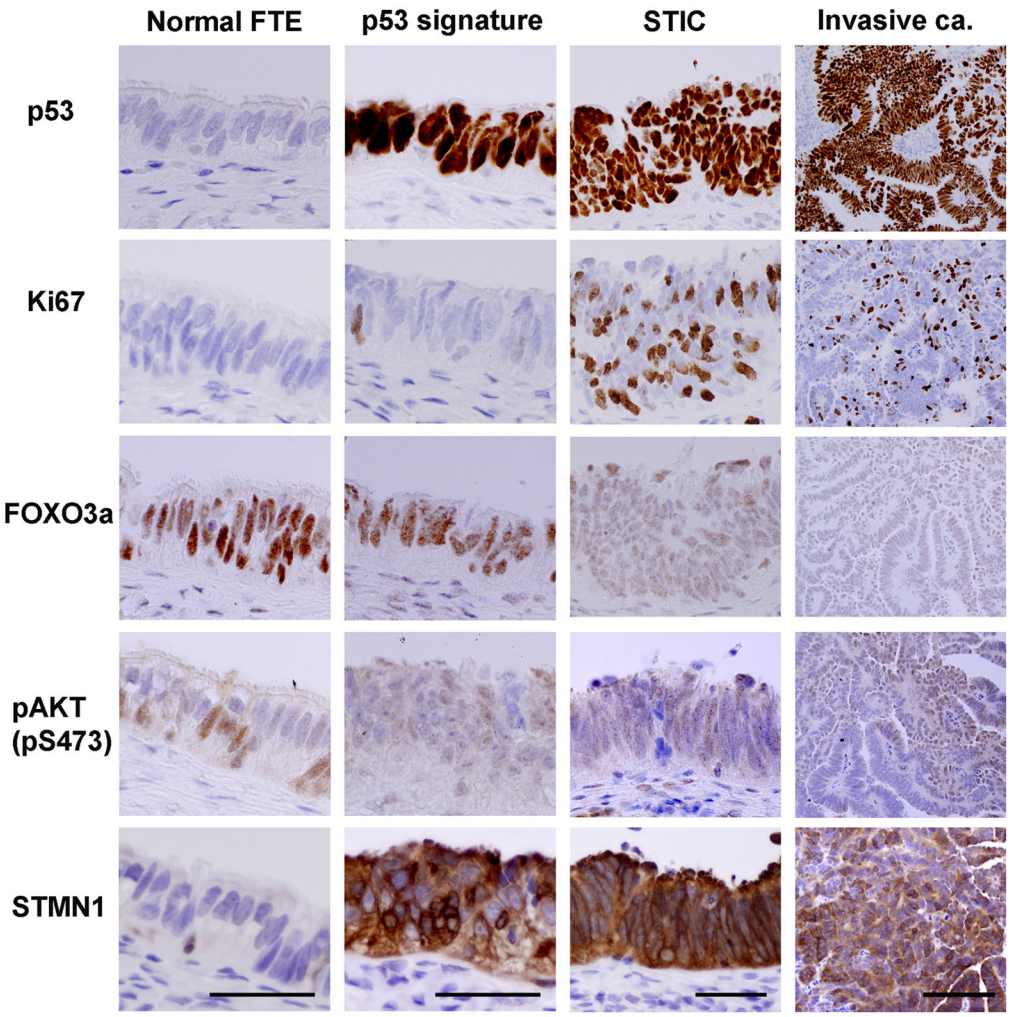

Figure 4. Activation of PI3K pathway is an early event in serous carcinogenesis

Immunostaining of normal FTE, 'p53 signature', STIC and invasive serous carcinoma from a single patient indicating that the PI3K pathway is aberrantly activated at the 'p53 signature' phase. Staining for phospho-AKT (pS473) is not sufficient to report the aberrant activation of the PI3K pathway, which is demonstrated by strong staining for stathmin (STMN1) at the 'p53 signature', STIC, and invasive carcinoma stages. Bar - 50 μm.

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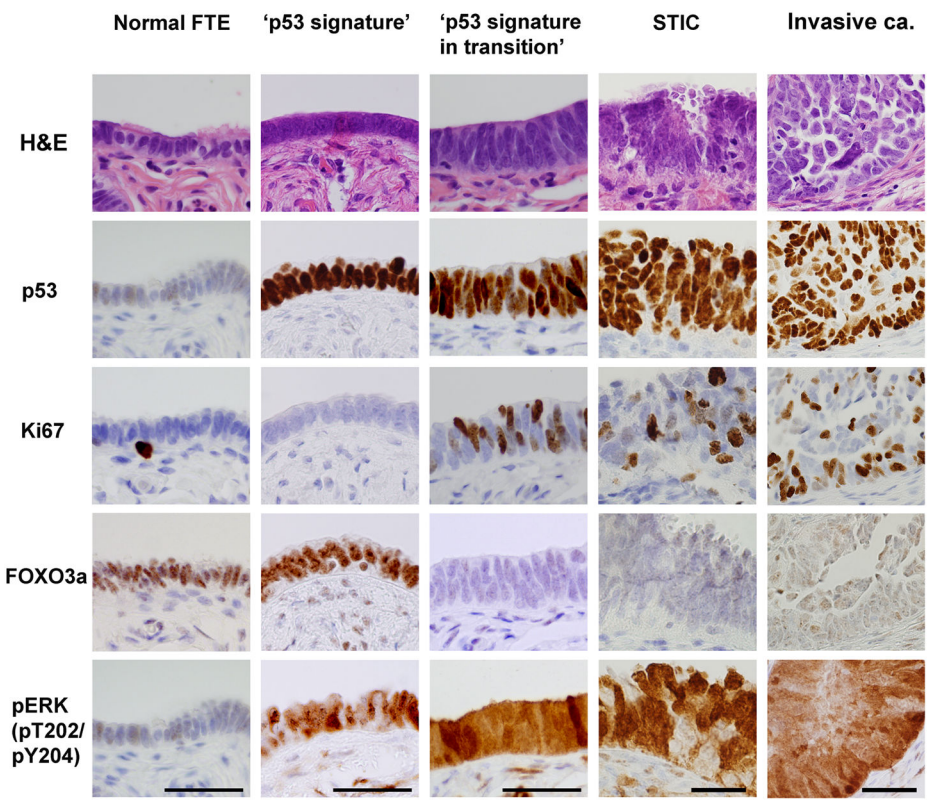

Figure 5. Activation of RAS/MEK/ERK pathway is an early event in serous carcinogenesis

H&E and immunostaining of normal FTE, 'p53 signature', 'p53 signature in transition', STIC and invasive serous carcinoma from a single patient indicating that the ERK pathway is aberrantly activated at the 'p53 signature' phase. Staining for phospho-ERK (pT202/ pY204) demonstrates activation of MEK at the 'p53 signature', STIC, and invasive carcinoma stages. Bar - 50 μm.

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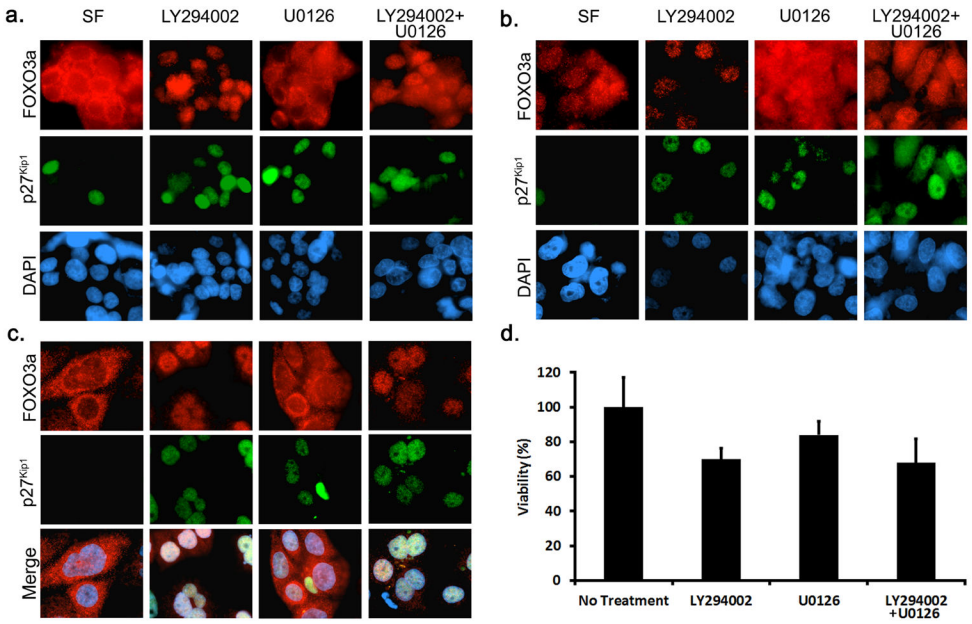

Figure 6. Effect of PI3K and MEK inhibitors on FOXO3a localization

Representative immunofluorescent images of ovarian carcinoma cell lines OVCAR3 (a), OVCAR8 (b) and primary tumor cells derived from patients' ascites fluid (c) with addition of LY294002, U0126, or a combination of both, for 24 hours. Cells were stained for FOXO3a (red), $p27^{kip1}$ (green), and the nuclei were counterstained with DAPI (blue). FOXO3a is predominantly localized to the cytoplasm in these cell lines, but translocates to the nucleus upon treatment with PI3K or MEK inhibitors. d. Viability assay of IGROV1 cells following treatment with LY294002, U0126, or a combination of both.