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TITLE: PDI Coamplified Genes in Ovarian Cancer

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REPORT DATE: August 2017

TYPE OF REPORT: Final

PREPARED FOR:

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE August 2017		2. REPORT TYPE Final		3. DATES COVERED 1Jun2014 - 31 May 2017	
4. TITLE AND SUBTITLE PDI Coamplified Genes in Ovarian Cancer				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH -14-1 -0172	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Nouri Neamati neamati@umich.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Michigan Ann Arbor, MI 48109				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Epithelial ovarian cancers (EOCs) are a heterogeneous group of tumors with distinct subtypes having different tissues of origin, diverse genetic landscapes, and respond differently to therapy.1-3 For example, serous EOC is thought to originate in the distal fallopian tube4 whereas ovarian clear cell carcinomas (OCCCs) originate from endometriotic tissues.5 While mutations in p53 are uncommon in OCCCs (9-10%) they are common in serous EOC (96%).6 OCCCs are usually classified as high-grade carcinomas, and were considered a uniform entity. However, recent studies have revealed that OCCCs are genetically heterogeneous and can be further subdivided into distinct categories.7 In the United States, OCCCs account for 5-13% of all EOCs, whereas in Japan they account for 15-25%. OCCCs are associated with poorer prognosis and are frequently resistant to conventional platinum-based chemotherapy. We hypothesize that subgroups of EOC harbour specific genetic alterations that ultimately override the apoptotic machinery to render them more chemoresistant than other EOCs. Such genetic alterations are best studied in a broad panel of samples from different ethnicities.					
15. SUBJECT TERMS- Nothing listed					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			USAMRMC
			UU	15	19b. TELEPHONE NUMBER (include area code)

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Comments to PI after submission of the report

This report has data on tasks other than what is on the approved SOW for this award. TMA's on approved SOW include:

- 1) The TMA at UM constructed from 30 benign ovary specimens and 148 epithelial ovarian cancer specimens.
- 2) The Yokohama TMA constructed from samples consisting of 53 chemotherapy-naïve serous, 50 clear cell, 22 endometrioid, 12 mucinous adenocarcinoma cases and 12 malignant tumors of other histologic cases together with 4 tumors of borderline malignancies.
- 3) The USC TMA (20-50 cases) is made from Caucasians, African Americans, Hispanics, and Asians patient samples.

No animal TMA's were on your approved SOW, but are referenced in your report. Please revise accordingly and resubmit a revised report.

Answer:

1. The full description of our TMA samples is provided in the first manuscript that we are currently writing. I am attaching the first draft of this manuscript with this report. We plan to submit this manuscript to Clinical Cancer Research within the next few weeks.
2. We did not produce any animal TMA. We just made xenograft of the cell lines and performed IHC staining to optimize our TMA studies. To perform IHC on 20 different antibodies from different vendors it required extensive optimization and validations.

Please note:

Progress report dates: As communicated during the first-year progress report our IRB approval and final decision to begin the project took exactly one year. Therefore, we could not generate any data during the first year. This is a copy of our first-year progress report that goes beyond the period from 6-1-14 to 5-31-15.

Introduction: Epithelial ovarian cancers (EOCs) are a heterogeneous group of tumors with distinct subtypes having different tissues of origin, diverse genetic landscapes, and respond differently to therapy.¹⁻³ For example, serous EOC is thought to originate in the distal fallopian tube⁴ whereas ovarian clear cell carcinomas (OCCCs) originate from endometriotic tissues.⁵ While mutations in p53 are uncommon in OCCCs (9-10%) they are common in serous EOC (96%).⁶ OCCCs are usually classified as high-grade carcinomas, and were considered a uniform entity. However, recent studies have revealed that OCCCs are genetically heterogeneous and can be further subdivided into distinct categories.⁷ In the United States, OCCCs account for 5-13% of all EOCs, whereas in Japan they account for 15-25%. OCCCs are associated with poorer prognosis and are frequently resistant to conventional platinum-based chemotherapy. We hypothesize that subgroups of EOC harbour specific genetic alterations that ultimately override the apoptotic machinery to render them more chemoresistant than other EOCs. Such genetic alterations are best studied in a broad panel of samples from different ethnicities.

Our studies show that a particularly important candidate gene implicated in EOC heterogeneity and chemoresistance is the pro-oncogenic protein disulfide isomerase (PDI). PDI is a 57-kDa chaperone protein located in the endoplasmic reticulum (ER) encoded by *P4HB* gene. Few studies have examined PDI expression and activity in cancer. Current data suggest that overexpression of PDI and its select family members is linked to cancer progression and chemoresistance, although the specific mechanism remains unknown. For example, expression of a well-characterized PDI family member, ERp57, is linked to paclitaxel-resistance in ovarian cancer.⁸ Another PDI family member, Anterior Gradient 2 (AGR2), is up-regulated during ER-stress and facilitates tamoxifen-resistance in breast cancer patients.⁹ Interestingly, AGR2 and a related family member, AGR3, mediate cisplatin-resistance in ovarian cancer.¹⁰ Cumulatively, available evidence implies that over-expression of PDI or its family members is associated with drug resistance, making it an intriguing biomarker and novel drug target.¹¹⁻¹⁷ Our study will provide a first methodical evaluation of the expression of PDI and its family members in all subtypes of EOC in different ethnical backgrounds and will correlate their expression with prognosis, survival, and response to therapy. Successful completion of this proposal will validate PDI and additional genes as biomarkers and drug targets in subsets of EOCs. Moreover, these studies will further evaluate the mechanism of PDI in cancer and facilitate translation of our novel PDI inhibitors to the clinic.

HYPOTHESIS or OBJECTIVES: We hypothesize that differences in responsiveness to conventional therapies among the various subtypes of EOC are determined by a unique set of genetic alterations in these cancers. We further hypothesize that PDI amplification leads to co-amplification of other genes, and that a subset of these genes can serve as prognostic markers for selecting patients for the right treatment using conventional therapy and/or PDI inhibitors. The objectives of our studies are to 1) characterize the expression levels of PDI and additional nine coexpressed genes we have recently identified in EOC subtypes using cell lines and patient samples; and 2) determine if the expression of these genes correlate with either increased or decreased response to conventional therapies and our PDI inhibitors. We will compare the expression of these genes along with five additional PDI family members (PDIA2, AGR2, PDIA3, PDIA4, and PDIA6) and six genes implicated in the PDI and ER stress pathways (GRP78, PERK, IRE1, ATF4, ATF6, and CHOP) as reference genes. Therefore, the expression of a total of 20 genes will be analyzed.

SPECIFIC AIMS: **Aim 1.** Validate candidate genes that are co-amplified and over-expressed with PDI in ovarian cancers tissue specimens. Samples from distal fallopian tubes, ovaries, and endometriotic tissues will be used as normal controls. **Aim 2.** Determine how cells with low and high expression of PDI and co-expressed genes are differentially responsive to conventional therapies and our novel PDI inhibitors or PDI-knockdown in cultured cell lines. **Aim 3.** Validate the association of key genes with clinical outcome and response to treatment in retro- and prospective analyses of EOC patients.

Key Research Accomplishments

Task 1. Validate candidate genes that are co-amplified and over-expressed with PDI in ovarian cancers tissue specimens.

It took us one full year to get IRB approval, obtain TMA from three institutions, and get approval from the DoD to begin our work. While waiting to perform human subject studies we have validated all the antibodies and currently are staining the TMA slides.

Task 2. Determine how cells with low and high expression of PDI and co-expressed genes are differentially responsive to conventional therapies and our novel PDI inhibitors or PDI-knockdown in cultured cell lines.

During the first year we purchased several antibodies from multiple vendors and tested them in various ovarian cancer cell lines. We had to contact, test, and validate antibodies from 5 separate vendors. Not a single vendor had all the antibodies. These data are summarized in this report.

Task 3. Validate the association of key genes with clinical outcome and response to treatment in retro- and prospective analyses of EOC patients.

Having validated majority of the antibodies for TMA staining we will soon complete the rest of the studies.

Reportable Outcomes

Methods

Preparation of tumor lysate and Western blotting: Tumor tissues were collected from mouse xenograft models, half of the mass preserved for IHC staining, other half of the mass was flash frozen in liquid nitrogen for western blotting. Tissue samples were thawed in RIPA buffer (200 μ L to 400 μ L) supplemented with proteinase inhibitor and phosphatase inhibitor cocktail then homogenize with electrical homogenizer followed by short sonication to get homogeneous tissue lysate. Lysate solution was centrifuged at 12000 rpm for 30 min at 4⁰C. Protein concentrations of supernatants were measured with BCA assay (ThermoFisher Scientific). 30-40 μ g protein per sample was subjected to SDS-PAGE analysis. Proteins were then electro transferred to methanol activated immobilon-FL PVDF membranes (EMD Millipore, Billerica, MA). Membranes were blocked with starting block (ThermoFisher) for 1hr at room temperature. Membrane was incubated with primary antibodies for overnight at 4⁰C. Membranes were then washed with TBST (10min x 3), incubated with Dylight 800-conjugated secondary antibodies (ThermoFisher Scientific, Rockford, IL) 1:5000 dilutions in 5% milk for 1 h at room temperature, and washed with TBST (10 min x 2) and TBS (10 min). Fluorescent signal was then scanned by Odyssey Imaging Systems (LI-COR Biosciences, Lincoln, NE).

Tissue microarray (TMA) of mouse xenograft model: Tumor tissues were collected from mouse xenograft model and fix with 10% formalin for 24hr at room temperature. After fixation tissue samples were preserved in 70% ethanol and submitted to the pathology core to prepared TMA slide as well as paraffin embedded slide for IHC staining. Based on western blot results of the protein expression in the tumor sample from different xenograft a positive control for each protein was selected and antibody dilution were optimized for TMA. These antibody dilutions will be used for TMA staining for human ovarian cancer tissue specimens.

The following antibodies are not commercially available: LOC727967, PDIA2, and SLC52A2 and following antibodies were not suitable for IHC staining: ERN1, ATF4, and DDIT3.

Results

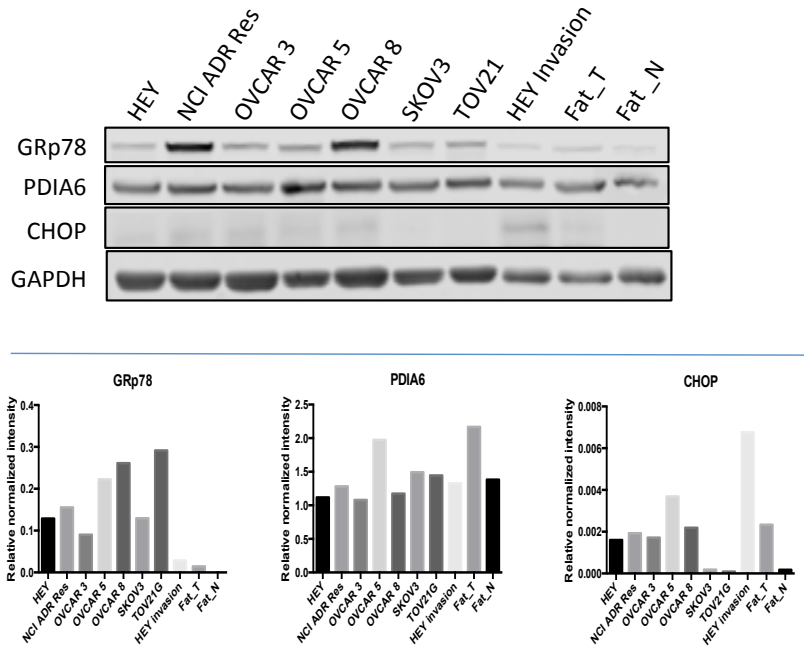


Figure 1. Expression of GRP78, PDIA6, and CHOP in a panel of ovarian cancer cell lines implanted in mice. HEY Invasion: HEY cells metastasis at distant sites. Fat T: adipose cells surrounding the tumor, FatN: normal adipose cells/tissues.

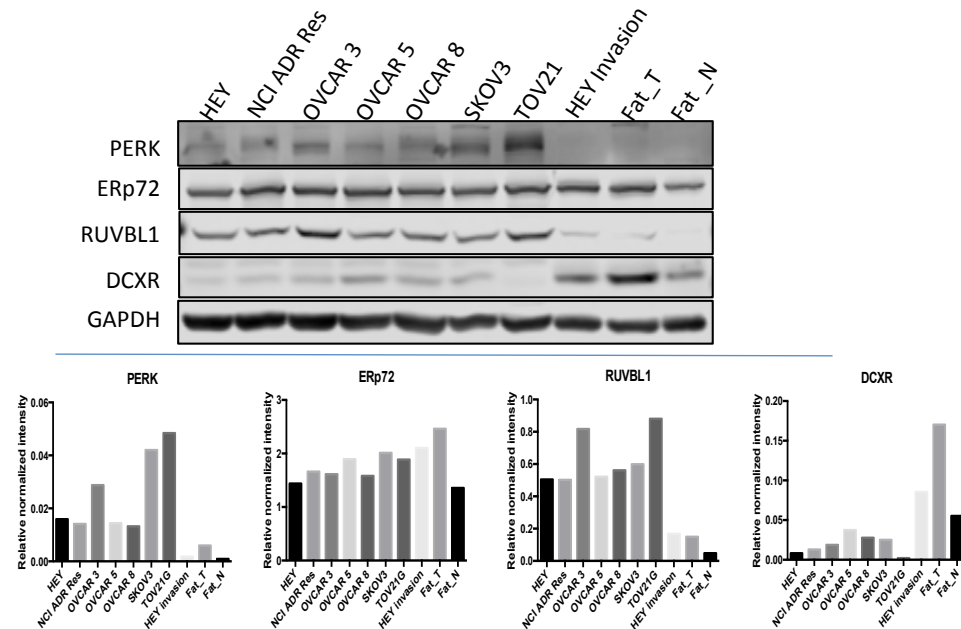


Figure 2. Expression and quantitation of PERK, ERp72, RUVBL1, and DCXR in a panel of ovarian cancer cell lines implanted in mice and normal adipose tissues obtained from the same mice.

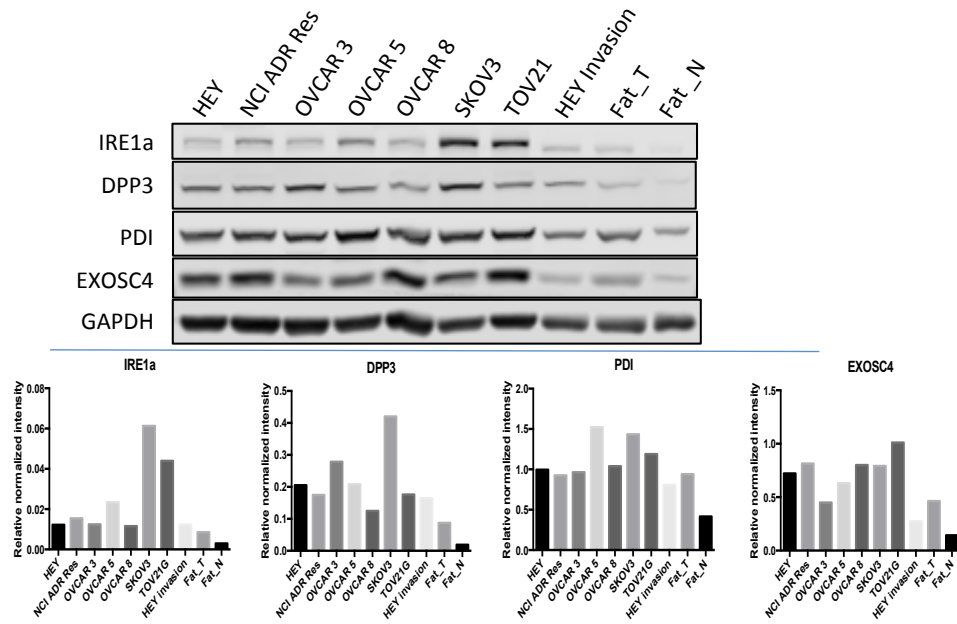


Figure 3. Expression and quantitation of IRE1a, DPP3, PDI, and EXOSC4 in a panel of ovarian cancer cell lines implanted in mice and normal adipose tissues obtained from the same mice.

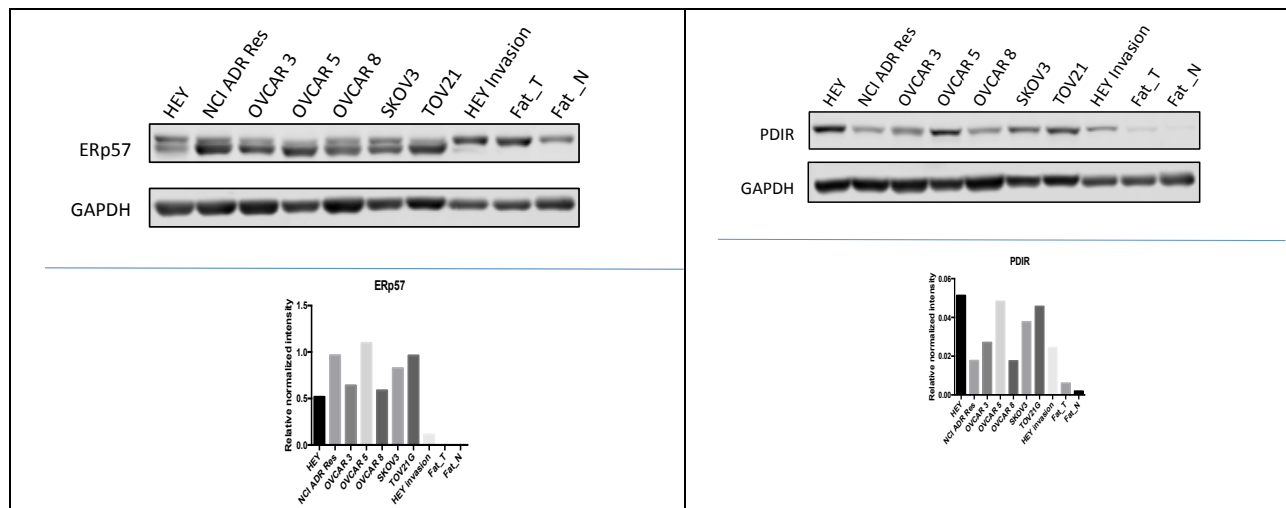


Figure 4. Expression and quantitation of ERp57 and PDIR in a panel of ovarian cancer cell lines implanted in mice and normal adipose tissues obtained from the same mice.

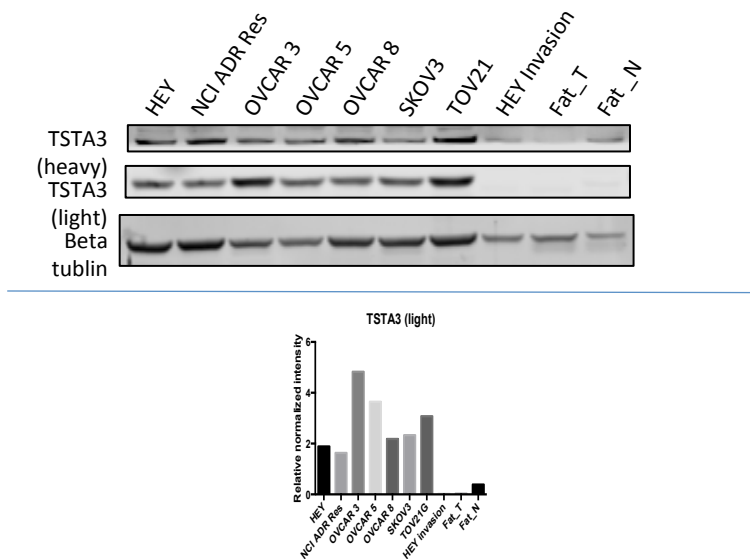


Figure 5. Expression and quantitation of TSTA3a and TSTA3b in a panel of ovarian cancer cell lines implanted in mice and normal adipose tissues obtained from the same mice.

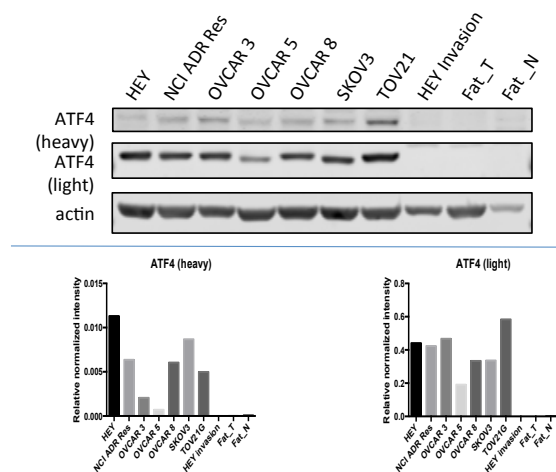


Figure 6. Expression and quantitation of ATF4 (heavy) and ATF4 (light chain) in a panel of ovarian cancer cell lines implanted in mice and normal adipose tissues obtained from the same mice.

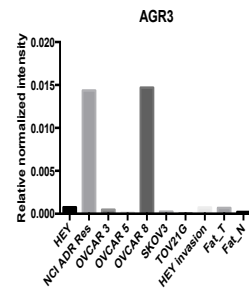
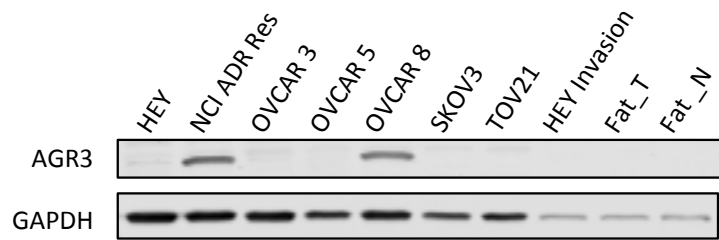


Figure 7. Expression and quantitation of AGR3 in a panel of ovarian cancer cell lines implanted in mice and normal adipose tissues obtained from the same mice.

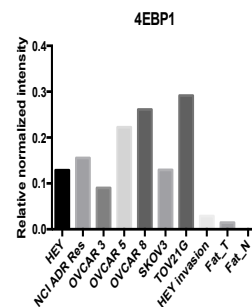
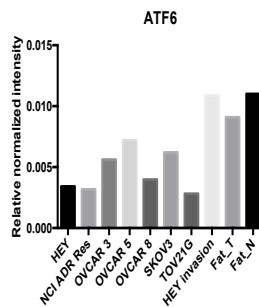
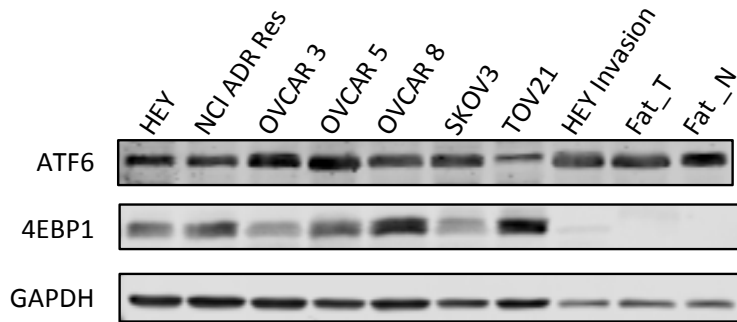
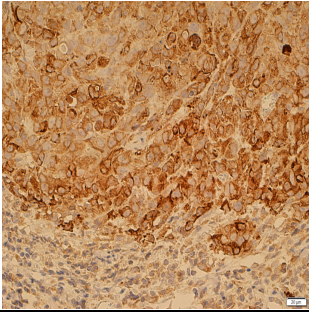
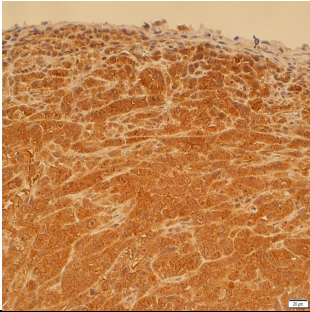
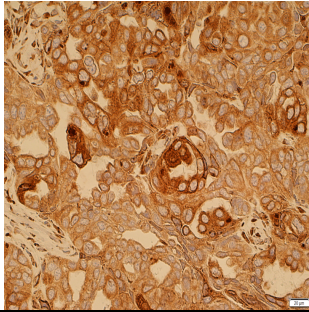
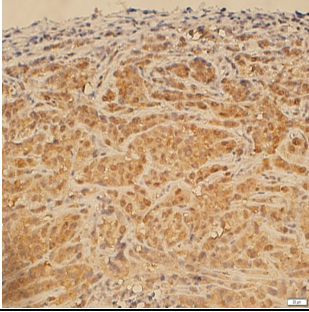
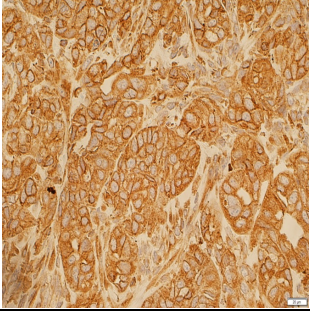
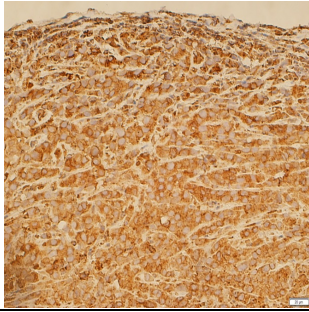
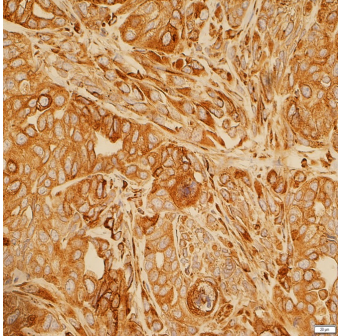
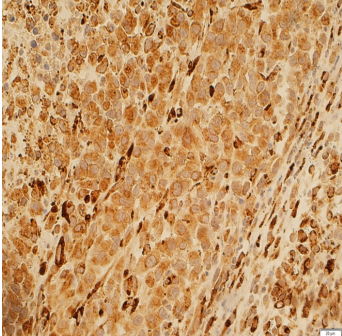
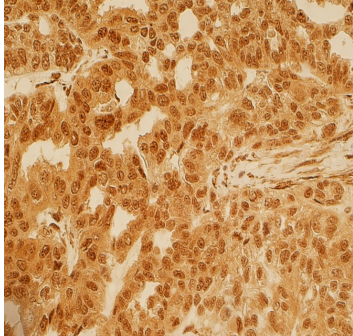
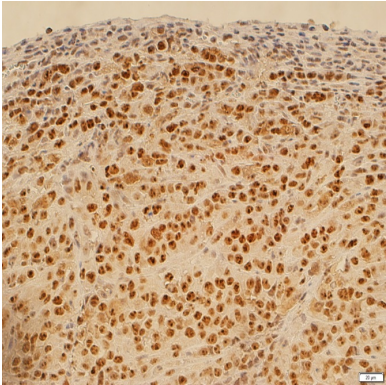
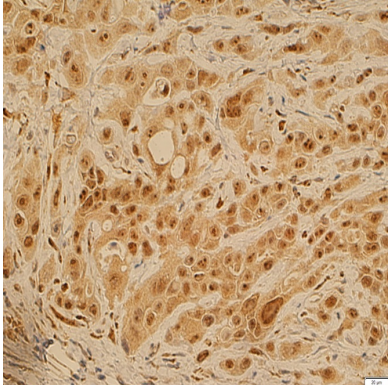
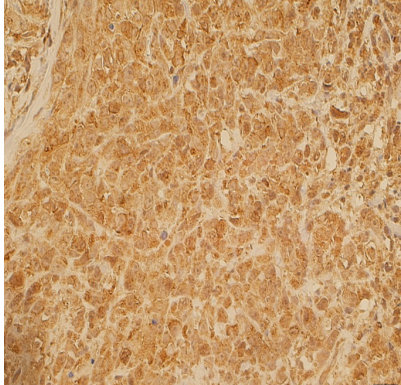
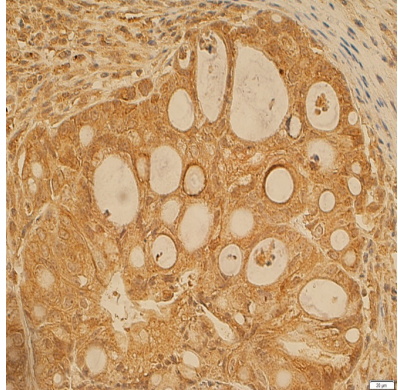
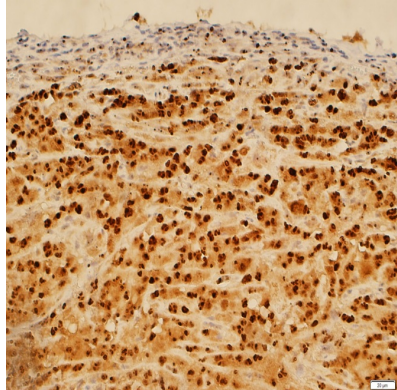
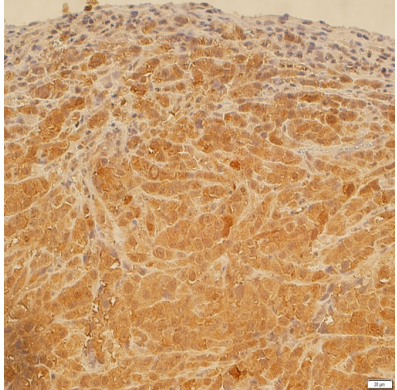
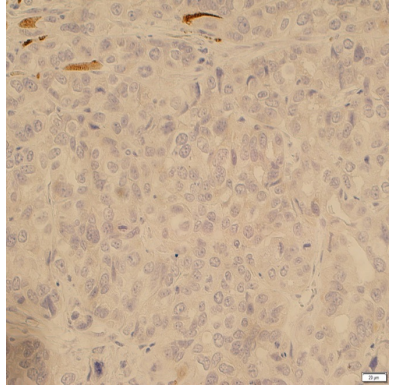
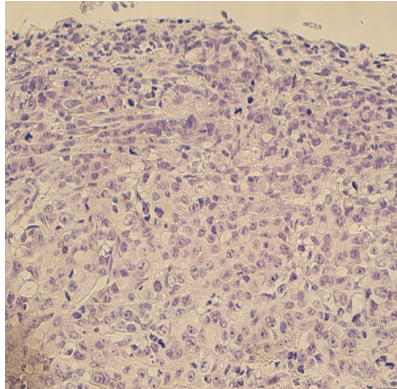
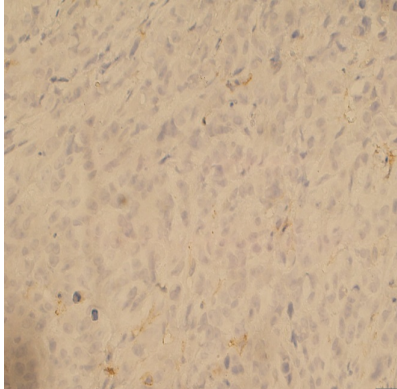


Figure 8. Expression and quantitation of ATF6 and 4EBP1 in a panel of ovarian cancer cell lines implanted in mice and normal adipose tissues obtained from the same mice.

Figure 9. IHC staining of select proteins in a mouse xenograft engrafted with ovarian cancer cell lines.

GRP78 	PERK 	PDI 
RUVBL1 	PDIA6 	ERp57 
ERp72 	PDIR 	DPP3 
EXOSC4 	ATF6 	AGR3 

DCXR 	TSTA3 	4EBP1 
IRE1a 	ATF4 	CHOP 

Conclusions: We have successfully tested a broad range of antibodies in cells implanted in mice (xenograft) models to establish a robust staining for analyzing three sets of tissue microarray. As predicted from our bioinformatics analysis we observed a clear overexpression of most of the proteins in ovarian cancer cell lines. The remaining task will include the completion of all TMA staining, in-depth analysis, and selection of important biomarkers for future clinical studies.

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