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13. SUPPLEMENTARY NOTES

14. ABSTRACT

For Aim 1, we demonstrated 1)The HSP90-inhibitors 17-AAG and AT13387 has single agent activity against CCNE1-amplified cell lines; 2)HSP90-inhibition downregulates homologous recombination (HR) DNA repair and downregulates expression of HR pathway genes; 3)The HSP90-inhibitor AT13387 synergizes with platinum against CCNE1-amplified cell lines.
For Aim 2, we demonstrated 1)FOXM1 is necessary for the survival of CCNE1 amplified epithelial ovarian cancer cells. 2)FOXM1 interacts with Rb in CCNE1 amplified epithelial ovarian cancer cells. 3)Characterized small molecule inhibitor that disrupts the interaction between FOXM1 and Rb in CCNE1 amplified epithelial ovarian cancer cells.
For Aim 3, we demonstrated 1) Certain miRNAs including miR-1255b, miR-148b*, and miR-193b* inhibit HR DNA repair 2) These miRNAs synergize with platinum against CCNE1-amplified cell lines, that is expression of these miRNAs sensitizes cells to platinum.

15. SUBJECT TERMS
Epithelial Ovarian Cancer, CCNE1 amplification, Homologous Recombination DNA Repair, Platinum analogues, MicroRNAs, Heat shock protein 90 (HSP90) inhibitors, Forkhead box protein M1 (FOXM1), Retinoblastoma (RB), Poly-ADP Ribose Polymerase Inhibitors (PARP-inhibitors)

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INTRODUCTION:

Approximately 20% of high grade serous ovarian cancers harbor Cyclin E1 (CCNE1) amplification and are associated with poor outcome and inferior responsiveness to standard platinum chemotherapy. Given their intrinsic resistance to platinum, management of CCNE1-amplified ovarian cancers is challenging. In this research, we evaluate three novel strategies against CCNE1-amplified ovarian cancers that address different aspects of CCNE1 biology. In the first aim, based on our preliminary data, we hypothesize that HSP90-inhibitors may be effective against CCNE1-amplified ovarian tumors because they suppress HR, downregulate BRCA1, and downregulate CCNE1. In the second aim, based on our preliminary data and the fact that RB functions downstream of cyclin E, we hypothesize that inhibition of FOXM1 and RB interaction is an effective approach for targeting CCNE1-amplified ovarian tumors. Specifically, suppression of FOXM1/RB interaction will lead to enhancement of RB/E2F interaction and suppression of E2F-dependent oncogenic activity resulting in activity against CCNE1-amplified cells. In the third aim, we hypothesize that miR-1255b, miR-148b*, and miR-193b* may be effective against CCNE1-amplified ovarian tumors in combination with platinum and PARPis. Potential mechanisms for this effect include suppression of HR and downregulation of BRCA1, RAD51 and BRCA2 that are relevant for CCNE1-amplified ovarian tumors which are dependent on hyperactive HR and are sensitive to suppression of BRCA1.

1. KEYWORDS:

Epithelial Ovarian Cancer, CCNE1 amplification, Homologous Recombination DNA Repair, Platinum analogues, MicroRNAs, Heat shock protein 90 (HSP90) inhibitors, Forkhead box protein M1 (FOXM1), Retinoblastoma (RB), Poly-ADP Ribose Polymerase Inhibitors (PARP-inhibitors)

2. ACCOMPLISHMENTS:

What were the major goals and objectives of the project?

The major goal for Aim 1 is to determine the activity of HSP90 inhibitors in CCNE1-amplified ovarian tumors.
The major goal for Aim 2 is to inhibit FOXM1 and RB interaction to suppress CCNE1-amplified ovarian tumors.
The major goal for Aim 3 is to determine the activity of certain miRNA mimics in combination with PARP-inhibitors or platinum against CCNE1-amplified ovarian tumors.

What was accomplished under these goals?

Since the starting of the award, substantial progress has been made toward achieving the goals as outline in the application.

For AIM 1:

During the first year of the award we were able to show that HSP90 inhibition downregulates homologous recombination (HR) DNA repair and that the HSP90-inhibitor AT13387 synergizes with PARP-inhibitors against CCNE1-amplified cell lines, including OVCAR4 and OVCAR3 which exhibit CCNE1 overexpression and amplification respectively.

During the 2nd year of the award we initiated our assessment of the effects of the combination of HSP90-inhibitors and PARP-inhibitors in patient derived CCNE1-amplified ovarian cancer cells that have been orthotopically propagated in immunocompromised mice which was a major goal of Aim 1.

Specifically, ovarian cancer cells have been taken from patients and implanted intraperitoneally into immunocompromised mice. These models have been luciferized thereby providing the ability to follow the growth of tumors and response to targeted therapies by non-invasive bioluminescent imaging (BLI) technology. Growth kinetics of each model have been accurately determined using the BLI technology which allows reproducibility of model growth and accurate estimation of tumor burden which is important for randomization prior to drug testing. In addition, surrogate biomarkers such as CA125 have been evaluated as alternative methods to assess tumor burden and response to therapy.

We initially performed tolerability studies in three ovarian PDX models (DF101, DF106, DF09) using the PARP-inhibitor olaparib and the HSP90-inhibitor AT13387. As shown in Figure 1, doses of olaparib up to 100mg/kg po daily x 3 weeks and AT13387 up to 45mg/kg po for 2 days (D1,D2) on / 5 days off x 3 weeks (i.e. Days 1, 2, 8, 9, 15, 16) were well tolerated without weight loss in the mice.

Efficacy studies were subsequently performed; the study design is shown in Figure 2 below. Olaparib was dosed at 100mg/kg po daily x 4 weeks, AT13387 was administered at 45mg/kg po for 2 days (D1,D2) on / 5 days off x 4 weeks (i.e. Days 1, 2, 8, 9, 15, 16, 22, 23).

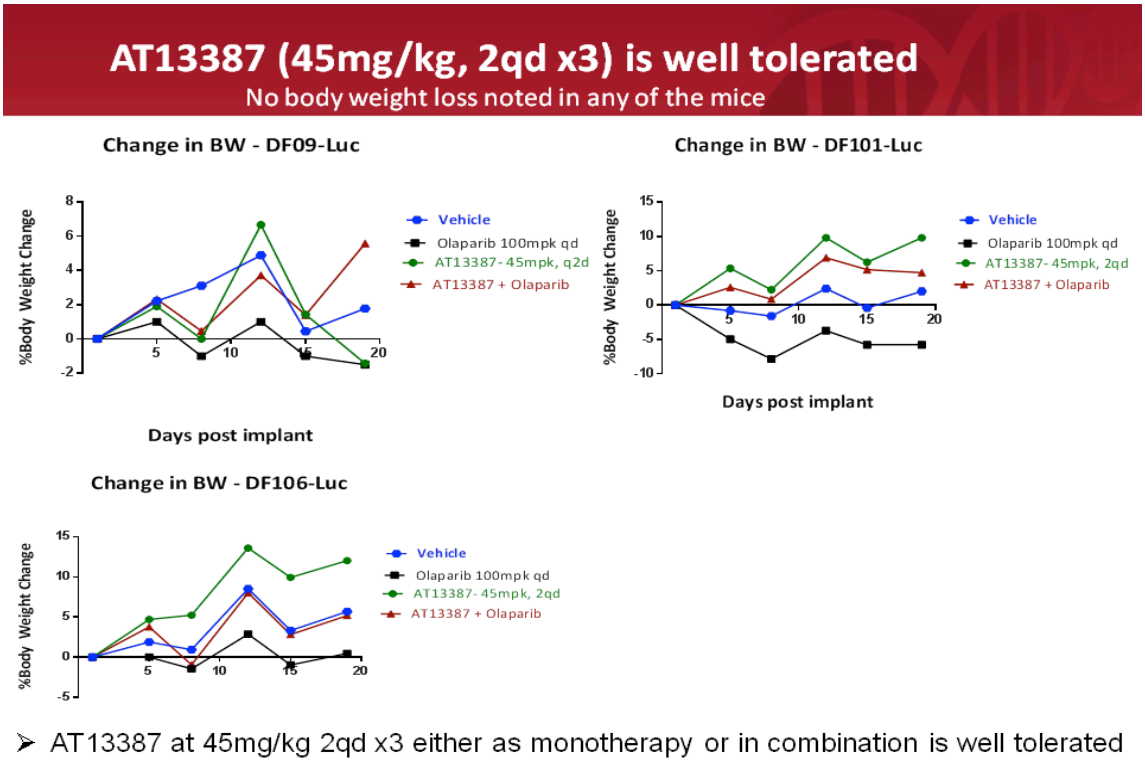

Figure 1. Tolerability of olaparib and AT13387 combination in ovarian PDX models.

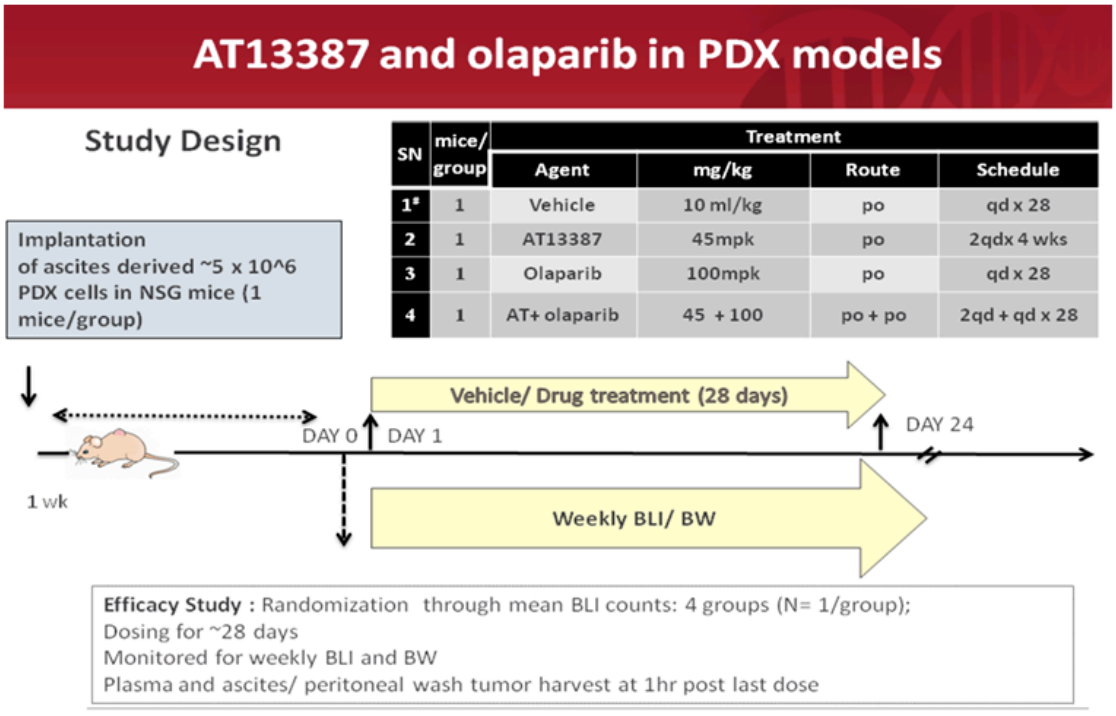

Figure 2. Design of efficacy studies of AT13387 and olaparib in ovarian PDX models.

Although we have not completed evaluation of all CCNE1-amplified models, evaluation of the DF-149 model which harbors CCNE1 overexpression was completed.
As shown in Figure 3, the combination of AT13387 and olaparib induced inhibition of tumor growth in the DF149 tumor model as opposed to vehicle control, olaparib alone and AT13387 alone.

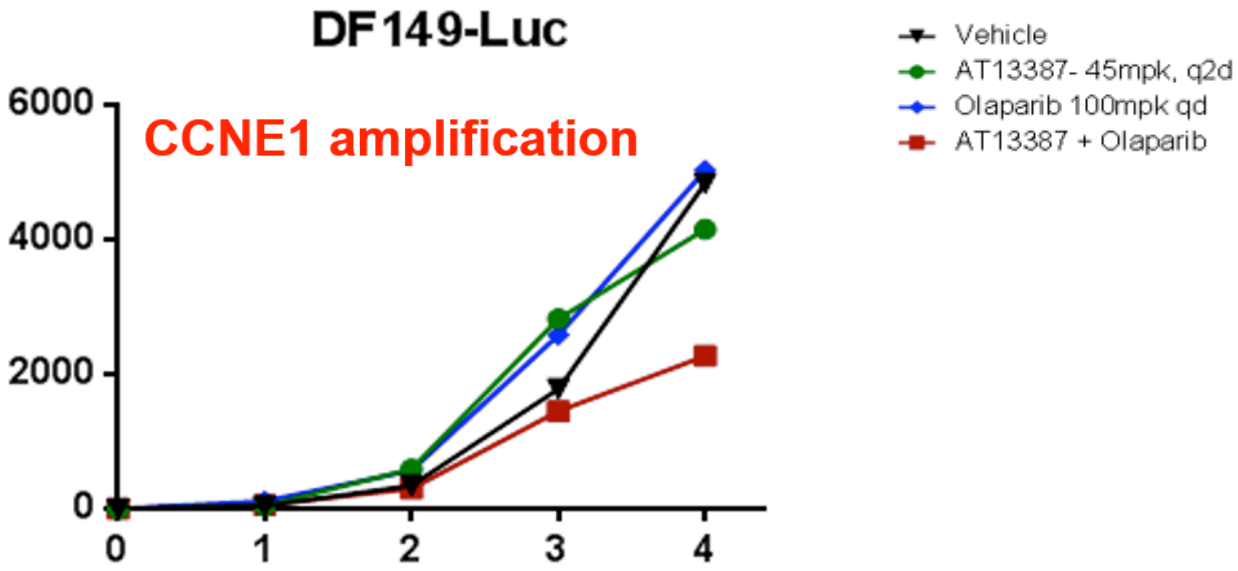

Figure 3. AT13387 and olaparib induced inhibition of tumor growth in the DF149 tumor model as opposed to vehicle control, olaparib alone and AT13387 alone

It is important to underscore that the DF-149 model was derived from patients with platinum resistant tumor. This result in the DF-149 supports our hypothesis of Aim 1 that HSP90-inhibitors may sensitize tumors to PARP-inhibitors.

For AIM 2:

During the first two years, we have made substantial progress. This aim focus on targeting FOXM1 dependence in *CCNE1*-amplfied EOCs. The retinoblastoma protein (RB) is a tumor suppressor that functions downstream of cyclin E1 (encoded by the *CCNE1* gene) to regulate cell cycle, apoptosis and differentiation through its direct binding to and inhibition of the E2F transcription factor [1, 2]. Disruption of RB and E2F interaction by viral oncogenic proteins such as HPV-E7 leads to neoplastic transformation [3]. HPV-E7 inhibits RB function through a conserved LxCxE motif for high affinity RB binding [3, 4]. Although RB pathway including its upstream regulator cyclin E is often deregulated in EOC [5], genetic alterations of the RB gene itself are relative rare in EOC [6-8]. Notably, RB physically interacts with FOXM1 [9, 10], a transcription factor with oncogenic activity in EOC [5]. Interestingly, the FOXM1 transcriptional network is significantly upregulated in EOC as well [5]. Therefore, we hypothesized that the interaction between RB and FOXM1 can be targeted in EOC. Since RB functions downstream of cyclin E, we expect that this approach will be especially effective in *CCNE1* amplified EOCs.

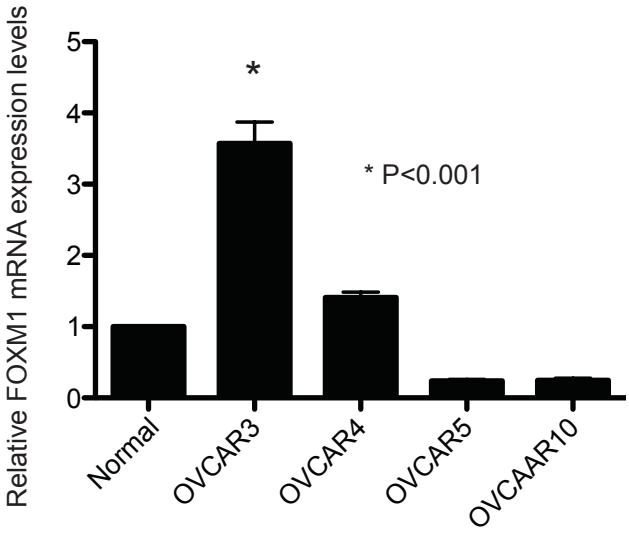

Figure 4. Expression of FOXM1 mRNA in a panel of EOC cell lines and normal fallopian tube epithelial cells determined by qRT-PCR.

We first examined the expression of *FOXM1* mRNA in a panel of EOC cell lines by qRT-PCR. We used normal fallopian tube epithelial cells as a control. Notably, *CCNE1*-amplified OVCAR3 showed the highest levels of FOXM1 expression (**Figure 4**). Thus, we used OVCAR3 cells to perform the subsequent functional studies. To determine the role of FOXM1 in OVCAR3 cells, we developed a shRNA targeting the human FOXM1 gene. We validated the knockdown efficiency fo shFOXM1 by both qRT-PCR and immunoblotting (**Figure 5**).

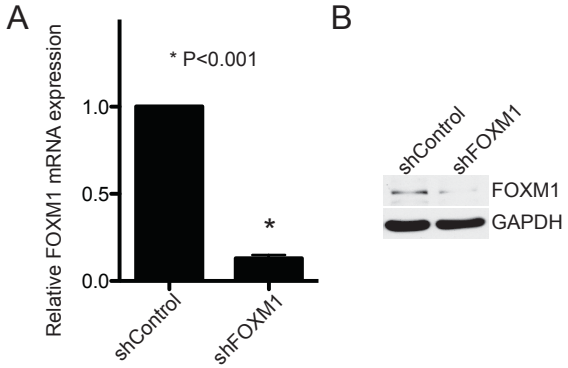

Figure 5. shRNA mediated knockdown of FOXM1 in OVCAR3 cells. Expression of FOXM1 in OVCAR3 expressing shFOXM1 or controls was determined by qRT-PCR (A) or immunoblotting (B).

Supporting the notion FOXM1 is required for the proliferation of CCNE1 amplified EOC cells. FOXM1 knockdown significantly suppressed the growth of OVCAR3 cells as determined by both cell growth curve and colony formation assays (**Figure 6**).

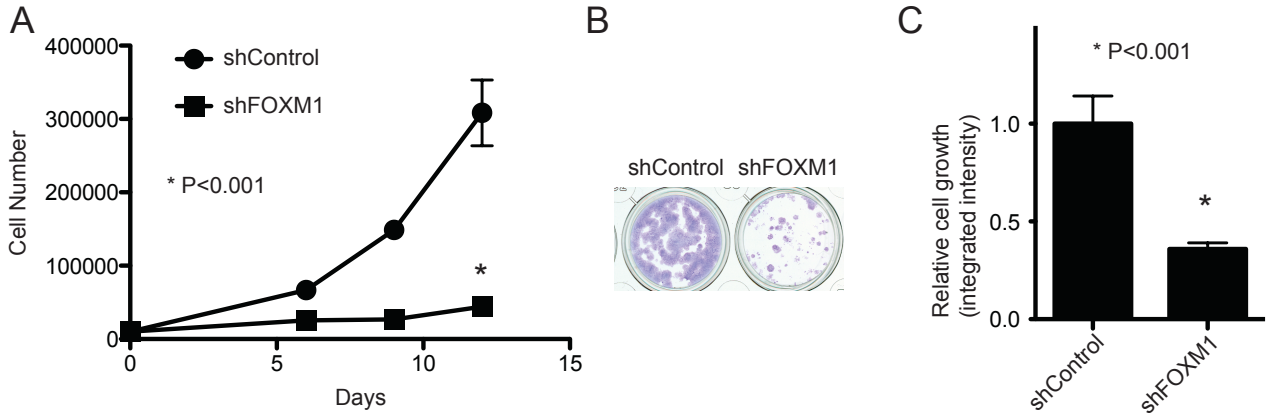

Figure 6. FOXM1 knockdown suppresses the growth of OVCAR3 cells. (A) Growth curves of OVCAR3 cells expressing shFOXM1 or controls. Mean of three independent experiments with SD. (B) Same as (A) but for colony formation assay. (C) Quantification of (B). Mean of three independent experiments with SD.

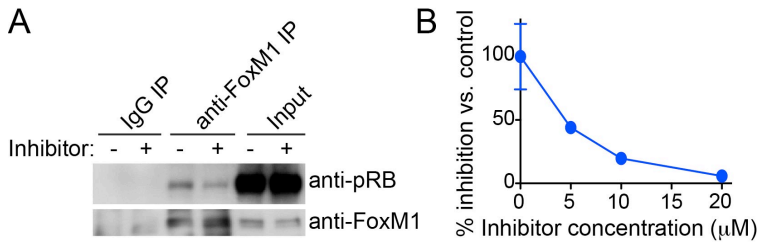

Figure 7. An inhibitor of FOXM1 and RB interaction that inhibits the growth of CCNE1 amplified NIH-OVCAR3 EOC cells. A) CCNE1 amplified NIH –OVCAR3 cells were treated with 5 mM inhibitor 478726 or control inactive compound 77333 for 48 h. The indicated cells were used for co-immunoprecipitation assay using an anti-FOXM1 antibody or a control IgG. The IPed product were examined by immunoblotting using the indicated antibodies. B) Same as A) but examined cell growth using the indicated concentration of inhibitor or control compound for 72 hours. Curves represent cell numbers normalized to control treatment.

The binding between FOXM1 and RB depends upon a LxCxE motif on FOXM1 [9, 10]. Notably, a class of thiadiazolidinedione compounds have previously been identified that disrupt the LxCxE motif mediated interaction between HPV-E7 and RB [11]. These compounds are selectively cytotoxic in HPV-positive cells *in intro* and *in vivo* in mouse models [11]. The observed effects correlate with its ability to suppress the disruption of RB/E2F complex by HPV-E7. This leads to a restoration of RB/E2F interaction and suppression of E2F dependent oncogenic activity. Since FOXM1's interaction with RB is also dependent upon the LxCxE motif [3, 4], we examined the effects of the RB/HPV-E7 disruption compound 478166 (or inhibitor 478726) on the interaction between FOXM1 and RB in EOC cells. In *CCNE1* amplified NIH-OVCAR3 cells [12], the interaction between FOXM1 and RB is readily detectable (**Figure 7A**). Co-immunoprecipitation analysis revealed that the interaction between FOXM1 and RB is substantially suppressed by inhibitor 478726 (**Figure 7A**). Since the disruption of interaction between HPV-E7 and RB by the inhibitor leads to cell growth arrest in HPV positive human cancer cells [11], we examined whether disruption of FOXM1 and RB also inhibits the growth of *CCNE1* amplified NIH-OVCAR3 cells. Indeed, we observed a dose dependent suppression of cell growth by the inhibitor

478726 in these cells (**Figure 7B**). In summary, our preliminary data identified a small molecule inhibitor that can disrupt the interaction between FOXM1 and RB, which correlates with a dose-dependent growth inhibition in *CCNE1* amplified EOC cells.

In summary, we have demonstrated in Aim 2:

1) FOXM1 is necessary for the proliferation of CCNE1 amplified epithelial ovarian cancer cells.
2) FOXM1 interacts with Rb in CCNE1 amplified epithelial ovarian cancer cells.
3) Characterized small molecule inhibitor that disrupts the interaction between FOXM1 and Rb in CCNE1 amplified epithelial ovarian cancer cells.

For AIM 3:

In the first year we observed (i) that miR-1255b, miR-148b*, and miR-193b* mimics suppress HR in CCNE- amplified ovarian cancer cell lines (ii) they work in combination with PARP-inhibitors or platinum against CCNE1-amplified ovarian cancer cell lines. The two issues we addressed in these year was to explore the in vivo relevance of the miRNAs. We generated OVCAR4 cells stably over-expressing these miRNAs from a chimeric construct and confirmed that these cells were hypersensitive to olaparib. Mice with xenografts of OVCAR4 cells expressing miRNAs or the scrambled control were treated with olaparib at a dose that typically does not impede tumor growth (Fig. 8, left panel). As anticipated the control tumors did not respond to olaparib but tumors expressing the miRNAs had a significant reduction in tumor volume (Fig. 8, right panel). The impact of olaparib on tumors expressing miRNAs was further confirmed by staining for caspase 3 activity (marker for apoptosis) and Ki67 (marker for proliferation). There was a clear increase in apoptotic activity and a decrease in proliferation in response to olaparib only in tumors derived from miRNA expressing cells (Fig. 9). These results strongly indicate that miR-1255b, miR-148b*, and miR-193b* sensitize CCNE- amplified ovarian cancer cell cells to PARP inhibitors both *in vitro* and *in vivo*, and there is potential for using them as therapeutic agents.

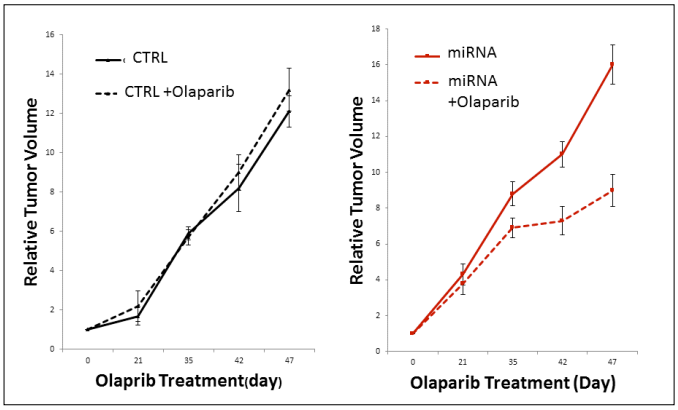

Figure 8. miRNA-1255b , miR-148b* and miR-193b* impact sensitivity to olaparib in orthotopic xenograft mouse models. Ovcar4 cells stably expressing control miRNA or miR-1255b-148b* were surgically implanted in nude mice and vehicle or PARP inhibitor treatment was initiated upon reaching tumor size of 200 mm^3 (n=20). Tumor volume was determined in 7 day-intervals and relative tumor volume was represented graphically, mean ± SD of all animals in each group.

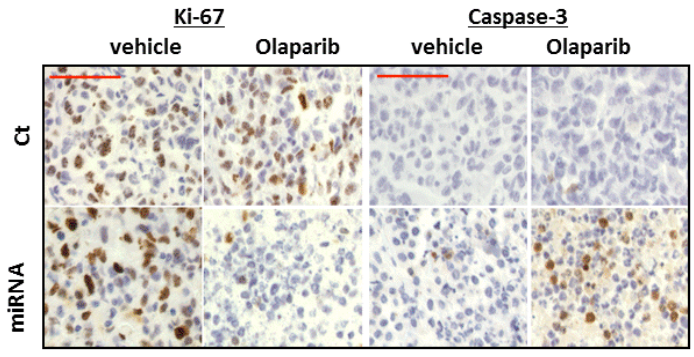

Figure 9. Tissues from control- or miRNA-expressing tumors with or without PARP inhibitor were stained for Ki-67 or caspase-3 to measure proliferation or apoptosis, respectively, in the tumors. Scale bar indicates 50 um.

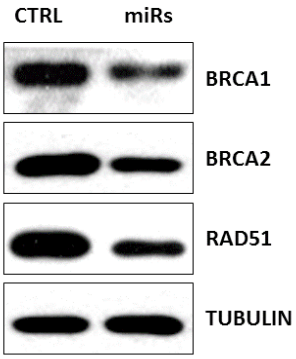

Figure 10. Tissues from control- or miRNA-expressing tumors were isolated and lysed in SDS-loading buffer. Immunoblot was conducted using the indicated antibodies.

In preliminary studies we had determined that that miRNAs, miR-1255b, miR-148b*, and miR-193b* target the DNA repair factors BRCA1, BRCA2 and RAD51. Next we investigated whether the targets of the miR-1255b, miR-148b*, and miR-193b* that were determined in preliminary studies remains unaltered in the xenografts. The tumors were retrieved from the animals and immunoblot conducted (Figure 10). Consistent with our previous results and the impact of the miRNAs on olaparib sensitivity, there is indeed an impact of the miRNAs on the levels of BRCA1, BRCA2 and RAD51.

We will continue these studies with additional CCNE-amplified lines and other PARP inhibitors. The other goal would be to express the miRNAs in patient derived xenograft.

What opportunities for training and professional development did the project provide?

During the 2nd year of the project Dr Konstantinopoulos was promoted to Associate Professor of Medicine at Harvard Medical School and was named Director of Translational Research in the Gynecologic Oncology Program at Dana-Farber Cancer Institute.

How were the results disseminated to communities of interest?

"Nothing to Report."

What do you plan to do during the next reporting period to accomplish the goals and objectives?

In the next reporting period:

For Aim 1, we plan to perform complete the assessment of the HSP90-inhibitor AT13387 and the PARP-inhibitor olaparib in all our ovarian PDX models. We also intend to conduct further synergism studies of HSP90-inhibitors with additional PARP-inhibitors and with platinum agents in CCNE1-amplified ovarian cancer cells.
For Aim 2, We will investigate the mechanisms by which FOXM1 suppression inhibits the growth of CCNE1-amplified cells. Specifically, we would like to explore whether the observed growth inhibition was due to apoptotic cell death or cellular senescence. In addition, we plan to extend these studies *in vivo*.
For Aim 3, we will continue these studies with additional CCNE-amplified lines and other PARP inhibitors. The other goal would be to express the miRNAs in patient derived xenograft.

3. IMPACT:

"Nothing to Report."

What was the impact on the development of the principal discipline(s) of the project?

"Nothing to Report."

What was the impact on other disciplines?

"Nothing to Report."

What was the impact on technology transfer?

“Nothing to Report.”

What was the impact on society beyond science and technology?

“Nothing to Report.”

4. CHANGES/PROBLEMS:

“Nothing to Report.”

Changes in approach and reasons for change

“Nothing to Report.”

Actual or anticipated problems or delays and actions or plans to resolve them

“Nothing to Report.”

Changes that had a significant impact on expenditures

“Nothing to Report.”

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

“Nothing to Report.”

5. PRODUCTS:

Journal publications.

1. Choi YE, Meghani K, Brault ME, Leclerc L, He YJ, Day TA, Elias KM, Drapkin R, Weinstock DM, Dao F, Shih KK, Matulonis U, Levine DA, **Konstantinopoulos PA, Chowdhury D**. Platinum and PARP Inhibitor Resistance Due to Overexpression of MicroRNA-622 in BRCA1-Mutant Ovarian Cancer. Cell Rep. 2016 Jan 26;14(3):429-439.
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Books or other non-periodical, one-time publications.

“Nothing to Report.”

Other publications, conference papers, and presentations.

“Nothing to Report.”

- **Website(s) or other Internet site(s)**

“Nothing to Report.”

- **Technologies or techniques**

“Nothing to Report.”

- **Inventions, patent applications, and/or licenses**

“Nothing to Report.”

- **Other Products**

“Nothing to Report.”

6. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:

What individuals have worked on the project?

Name:	*Rugang Zhang*
Project Role:	*Principal Investigator*
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1
Contribution to Project:	Supervised the study.
Funding Support:	This award

Name:	*Sergey Karakashev*
Project Role:	*Postdoctoral Fellow*
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	*5*
Contribution to Project:	Performed the study.
Funding Support:	This award

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

"Nothing to Report."

What other organizations were involved as partners?

"Nothing to Report."

7. SPECIAL REPORTING REQUIREMENTS: None

8. APPENDICES: None

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