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14. ABSTRACT

Epithelial ovarian cancer (EOC) is the most lethal gynecologic malignancy in the United States. One major obstacle in the clinical management of the disease is the high incidence of recurrence after cytotoxic chemotherapy and the development of platinum resistance. Given the crucial importance to overcome chemotherapy resistance to platinum therapy, we hypothesize that miRNA profiling in EOC cell lines and surgical specimens with varying chemosensitivities will uncover a potential predictive "fingerprint" for individualized therapy, while further biological validation of these miRNAs signatures will allow for the development of novel therapeutic strategies to enhance chemosensitivity. Through mircoarray analysis of miRNAs differentially expressed in an *in vitro* model of acquired carboplatin resistance consisting of EOC cell lines sensitive to carboplatin, A2780, and its resistant variants, CP20 (moderately resistant) and CP70 (resistant), we identified a panel of miRNAs that correlate with carboplatin response. We uncovered four miRNAs (miR-23b, miR-132, miR-183. miR-181a and miR-203) that are significantly upregulated in both platinum-resistant cell lines. Additionally, we found that miR-181a was also correlated with several clinical parameters in a cohort of ovarian tumor specimens from women diagnosed with stage III, grade 3, papillary serous adenocarcinoma all treated with platinum-based chemotherapy. Furthermore, we uncovered the biological relevance of this miRNA. We found that miR-181a induced platinum-resistance through the maintenance of cancer stem cells thourgh the regulation for TGFb and Wnt signaling pathway. In summary, in the past year we were able uncover the functional relevance of miR—181a (Task 2) and reveal that targeting this miRNA as a novel therapeutic option in ovarian cancer (Task 3)

15. SUBJECT TERMS

microRNA, ovarian cancer, platinum resistance

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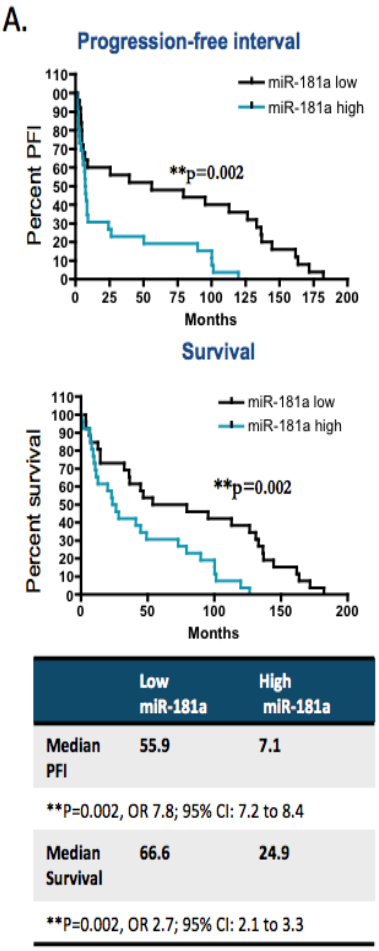
Introduction

Epithelial ovarian cancer (EOC) is the most lethal gynecologic malignancy. Platinum and taxane-based drugs are used in combination as first-line chemotherapeutics for women newly diagnosed with EOC, unfortunately approximately 20% do not respond to this treatment and have very poor outcomes due to chemoresistance, making advanced ovarian cancer particularly difficult to eradicate. Given the crucial importance to overcome chemotherapy resistance to platinum therapy, we hypothesize that miRNA profiling in EOC cell lines and surgical specimens with varying chemosensitivities will uncover a potential predictive "fingerprint" for individualized therapy, while further biological validation of these miRNAs signatures will allow for the development of novel therapeutic strategies to enhance chemosensitivity. The overarching goal of this project is to identify miRNAs involved in the development of ovarian cancer chemoresistance and ascertain their biological relevance using both *in vitro* and *in vivo* models of ovarian adenocarcinoma. To test our hypothesis, we propose to: 1) Validate the clinical relevance of the miRNA signature we identified in our *in vitro* model of chemosensitivity using a well-defined clinical cohort of ovarian cancer specimens; 2) determine the biological and functional relevance of the miRNAs that correlate with chemotherapeutic response**;** 3) explore whether *in vivo* targeting of miRNAs that are overexpressed in chemoresistant cancer cells can sensitize chemoresistant ovarian tumors to platinum treatment and inhibit ovarian cancer dissemination in a pre-clinical ovarian cancer mouse model. Our study, if successful, will identify key miRNAs involved in the regulation of chemoresistance in EOC and significantly contribute to the understanding of their biology and function *in vitro* and *in vivo*. Those miRNAs may also serve as predictive markers for tailored therapy. Furthermore, targeting these miRNAs using *in vivo* will provide a new paradigm for overcoming chemotherapy resistance clinically and thus improve survival in late stage disease.

BODY

To date, there have been only a few functionally relevant miRNA's identified in HGSOC and our studies supported by this Pilot Award uncovered a several highly relevant miRNAs that correlate with patient outcome and is enriched in platinum-resistant recurrent tumors. Through miRNA array analysis of an *in vitro* model of acquired platinum resistance consisting of an OvCa cell line sensitive to carboplatin, A2780, and its resistant variants, CP20 (moderately resistant) and CP70 (resistant) which, we have identified a panel of miRNAs that are correlated with carboplatin response (in previous progress report). Interestingly, the majority of miRNAs were upregulated in the platinum resistant variants compared to the parental sensitive cell line. Well-known miRNA clusters such as the miR-17-92 and the let7 family were some the most significantly altered miRNAs. Additionally, we both the moderately resistant and resistant cell lines had many of the same dysregulated miRNAs with only a few miRNA that were exclusively changed in each cell line alone .

As part of **Task 1** we proposed to validate the clinical relevance of the panel of miRNA's identified in our in vitro model of chemosensitivity using a well-defined clinical cohort of ovarian cancer specimens. Given that none of the preliminary miRNAs identified correlated with outcome we continued to expand our clinical cohort and examine the expression of other miRNAs that correlated with platinum-resistance. Upon expansion of our clinical cohort we uncovered a novel miRNA, miR-181a, correlated with patient outcome and tumor recurrence (**Figure 1A & B**). MicroRNA-181a expression dichotomized into miR-181a-high or miR-181a-low expressing tumors based on median expression in a clinically annotated patient cohort of high-grade stage III primary papillary serous ovarian cancer, revealed significant differences in the median progression-free survival (PFS) (59.9 months) for patients with low miR-181a expression, compared to 7.1 months in patients with high miR-181a expression (**Figure 1A**, *P*=0.002, OR 7.8; CI: 7.2-8.4). Furthermore, patients with low miR-181a levels exhibited a median overall survival (OS) of 66.6 months in contrast to a median OS of 24.9 months in patients with high miR-181a expression (**Figure 1A**, P=0.002, OR 2.7; CI: 2.1-3.3). Additionally, given that a major contributor to the overall poor survival of patients with high-grade serous ovarian cancer is tumor recurrence, we also assessed whether miR-181a was differentially expressed in recurrent tumors compared to matched-primary tumors. The expression of miR-181a was assessed in patient-matched tumor biopsies that were taken at primary surgery (PS-O: chemotherapy naïve ovarian tumor) and at secondary surgery (SCR: after tumor has recurred after at least two lines of chemotherapy). In the SCR tumors, miR-181a expression was upregulated 2.51-fold compared to PS-O (*P*=0.0006) (**Figure 1B**). Given the crucial importance of miRNAs in the regulation of global gene expression and cancer-relevant signaling pathways, our initial hypothesis was that a number of miRNAs play a yet unappreciated role in HGSOC development and chemotherapy resistance.



	Low miR-181a	High miR-181a
Median PFI	55.9	7.1
**P=0.002, OR 7.8; 95% CI: 7.2 to 8.4		
Median Survival	66.6	24.9
**P=0.002, OR 2.7; 95% CI: 2.1 to 3.3		

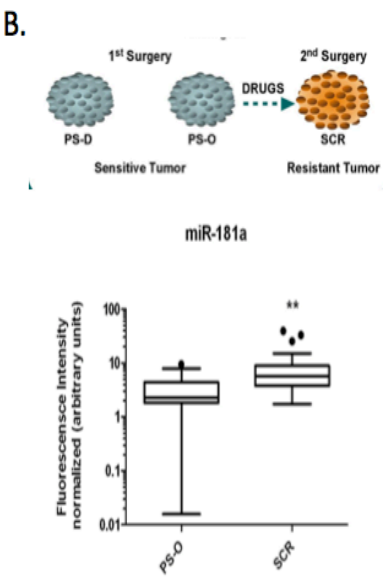


Figure 1: miR-181a levels are associated poor outcome and shorter time to recurrence in HGSOC. **(A)** Kaplan-Meier curves for progression-free survival (PFS) (top) and overall survival (OS) (bottom) according to relative expression levels of miR-181a (dichotomized at the median). **(B.)** Box plot diagrams showing the expression analysis of miR-181a in a cohort of patient matched tumor specimens from **PS-O**, primary surgery, ovary and **SCR,** secondary surgery.

Task 2: Determine the biological and functional relevance of the miRNAs that correlate with chemotherapeutic response.

Task 2 of our Statement of Work was focused on systemically validating the biological and functional consequences of dysregulated miRNAs that correlate with patient outcome in relevant HGSOC cell culture and in vivo models.

Through these studies, we found that miR-181a can affect key biological processes including tumor cell survival, chemotherapy response, cellular migration and invasion, and cancer cell dissemination *in vivo*[2]. Specifically, through the use of both intraperitoneal and intrabursal mouse models of ovarian cancer, we validated the functional relevance of miR-181a in ovarian cancer dissemination and progression in disease relevant in vivo models (**Figure 2**). Mice implanted with miR-181a expressing cells had increased overall tumor burden and a significantly higher incidence of metastatic nodules in the peritoneum (**Figure 2A**). Importantly, mRNA and IHC analyses of these tumors showed the maintenance of EMT *in vivo* (**Figure 2B**).

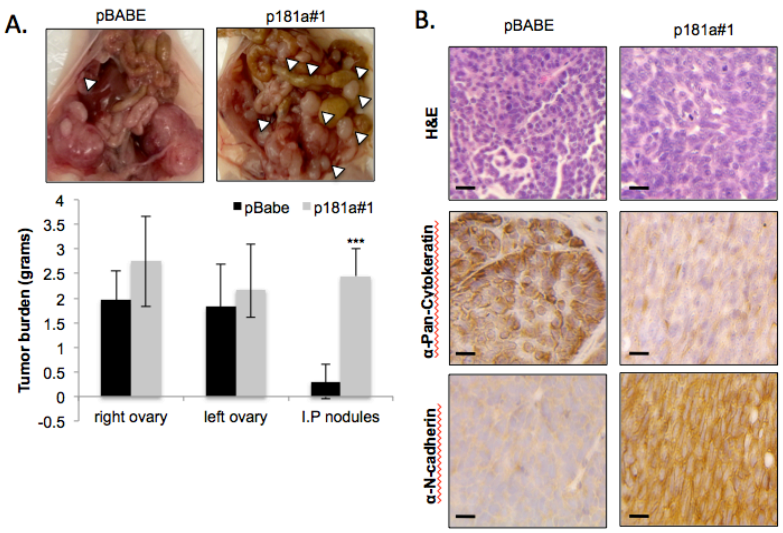


Figure 2. miR-181a regulates EMT and increases metastasis *in vivo*. **(a)** Gross images of metastatic nodules after intrabursal injection of ovarian cancer cells expressing pBABE control or pmiR-181a cells (top; white arrows). Ovarian tumors have been removed from the p181a#1 mouse in order to observe the extensive dissemination of tumor nodules throughout the abdomen. Total tumor burden in pBABE (n=5) vs. p181a-injected mice (n=7) at 3 weeks post-intraperitoneal injection ($2x10^6$ cells) (bottom). B. H&E and IHC staining(s) for pan-cytokeratin and N-cadherin in representative pBABE and p181a#1 tumors.

Following those studies we have found that miR-181a overexpression also enhances the stem-like features of HGSOC cells (**Figure 3**). We found that miR-181a expressing cells have increased expression of stem cell markers (**Figure 3B**). In addition, tumor sphere assays which allow for the assessment of whether a single cell harbors the potential to both initiate and maintain tumors in the absence of cellular interaction and adhesion, revealed that enhanced miR-181a expression significantly increased CICs (**Figure 3C**).

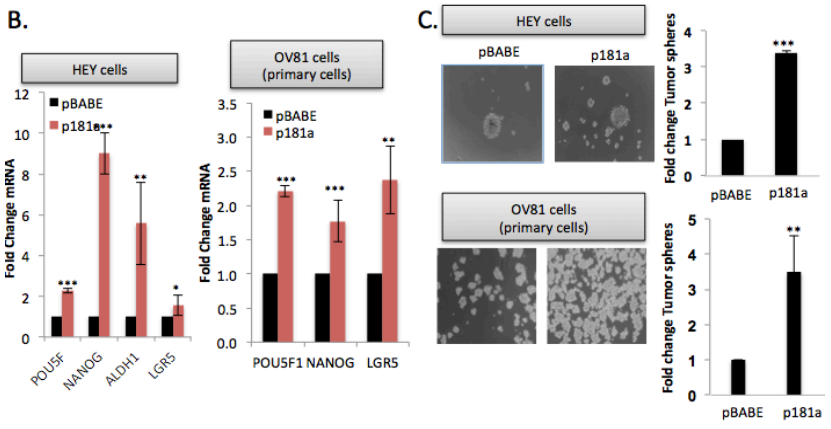
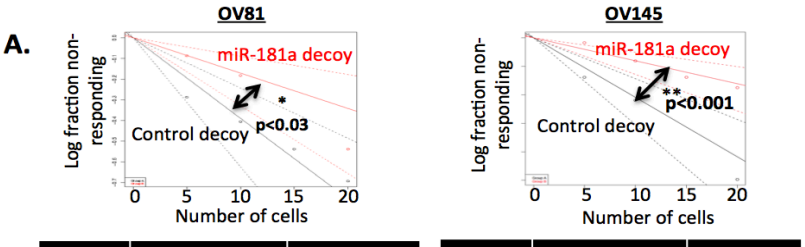


Figure 3: Enhanced miR-181a promotes EMT and contributes to the maintenance of CICs. **(B.)** qtRT-PCR of CIC markers in primary and established cells expressing miR-181a or control. **(C)** Tumor sphere assays in miR-181a and control cells. (tumor spheres were quantified using METAMORPH software and 100 random fields in 10x were counted).

Furthermore, we have now performed Extreme Limited Dilution Analysis [ELDA] in cells with targeted inhibition of miR-181a (miR-181a decoy) in order to assess miR-181a's direct effects on stem cell frequency. One, five, ten or twenty cells/ were sorted and placed directly into 96 well ultra-low attachment plates. After 7 days, the numbers of wells with tumor spheres were counted and the data was analyzed by ELDA platform. We found that miR-181a inhibition significantly decreased the stem cell frequency (**Figure 4A**). Lastly, we have also completed *in vivo* limiting dilution assays (LDA) and determined that miR-181a increased the tumor initiating properties of primary cells (**Figure 4B**).

Given that uncovering the molecular factors that contribute to the emergence of CICs is a critical component to understanding HGSOC drug resistance and recurrence we next sought to determine the mechanism by which miR-181a was regulating the emergence of these CICs.



	Average stem cell frequency	Confidence Interval
Control Decoy	1:25.7	16.0 to 41.1
miR-181a decoy	1:58.5	30.4 to 112.4

	Average stem cell frequency	Confidence Interval
Control Decoy	1:9.6	6.6 to 14.0
miR-181a decoy	1:24.9	15.6 to 39.6

B.

# tumor cells injected	# mice with tumors	
	p001	p181a
100,000	5/ 5	5/5
10,000	3/5	5/5*
1,000	1/5	5/5**

Figure 4: miR-181a contributes to the CICs. (**A**) ELDA and quantification of stem cell frequency in primary cells following control or miR-181a inhibition. **(B)** *In vivo* limiting dilution assay demonstrating increased tumor formation in nu/nu mice for p181a-expressing cells.

miR-181a upregulates Wnt/ βcatenin signaling

Emerging studies have delineated several ovarian CIC markers that have been used to isolated CIC from EOC cell lines and primary tumors. Most recently, the first stem-cell population that was able to give rise to ovarian cancers was identified in mice. Cells located between the ovary and tubal epithelium were found to express stem cell markers and displayed an increased ability to undergo transformation after inactivation *Trp53* and *Rb1*. Though numerous studies have focused on isolating candidate CIC populations, the biological characterization of the signaling pathways contributing to the emergence of ovarian CICs have yet to be defined. Several signaling pathways that are important to the generation and maintenance of normal stem cells during embryonic development [e.g., Notch, Wnt, and Hedgehog] are also important for the growth of many cancers. Members of the Wnt family have been shown to induce several distinct signaling events, including activation of the βcatenin signaling (termed the "canonical pathway") as well as other signaling cascades that are βcatenin independent. Ingenuity Pathway Analysis (IPA) of high-throughput mRNA sequencing from cells expressing miR-181a revealed that, in addition, to the TGFβ signaling pathway, the Wnt/βcatenin signaling pathway was also significantly altered (Fig. 5A). Interestingly, an integrated analysis of a large cohort HGSOC samples revealed that a WNT signaling signature was highly predictive of poor clinical outcome[14]. Though mutations in the Wnt pathway are rare in HGSOC increased βcatenin levels and/or altered βcatenin/Wnt signaling are commonly observed in ovarian cancer and aberrant accumulation of βcatenin correlates with poor survival[10-12]. Intriguingly, many cancers do not depend on mutations in key components of the pathway for activation of Wnt signaling[22-27]. Therefore we next sought to confirm whether miR-181a is regulating the Wnt/βcatenin pathway in HGSOC and could be a novel mechanism driving Wnt signaling. Figure 5, highlights some of the key findings validating that miR-181a is able to enhance the Wnt/βcatenin pathway. Given that the binding of a Wnt ligand to a receptor complex encompassing FZD1

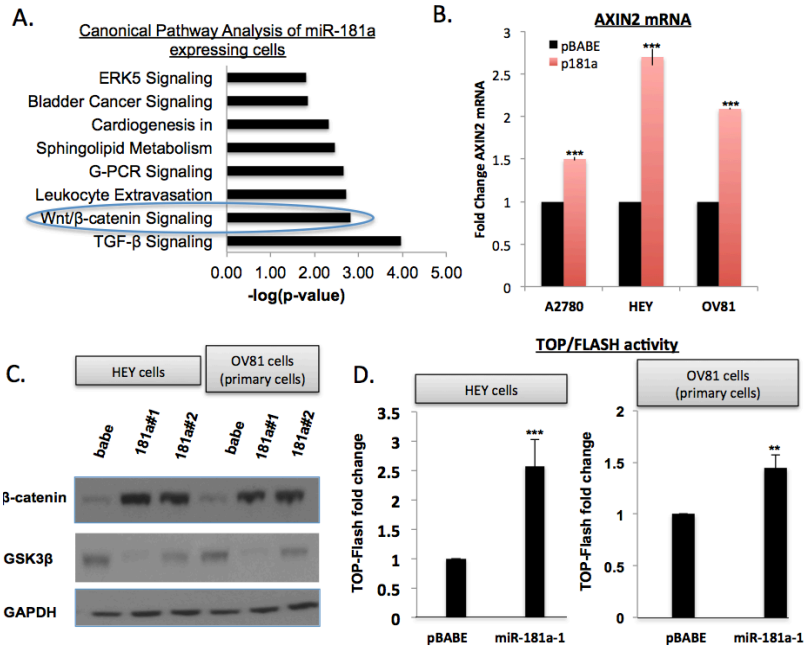


Figure 5: Enhanced miR-181a expression leads to the activation of the Wnt/ β-catenin. **(A)** Ingenuity Systems (IPA) analysis of miR-181a expressing cells revealed that Wnt/βcatenin canonical signaling pathways was significantly altered. **(B)** Induction of Axin2 in 3 HGSOC cells lines expressing miR-181a. **(C)** Cells expressing miR-181a display a stabilization of βcatenin protein and downregulation of GSK3β. **(D)** Activation of the Wnt/ βcatenin reporter in cells expressing miR-181a.

and a LRP coreceptor drives pathway activation resulting in the stabilization and nuclear βcatenin translocation, we first assessed the expression of activated βcatenin protein. We found that βcatenin is stabilized in miR-181a expressing cells and there is a concomitant decrease in GSK3β which regulates its degradation (Fig.5C). This stabilization resulted in an induction of downstream canonical WNT markers such as Lgr5 and Axin2 (the most universal output for Wnt signaling) (Fig. 5B). In order to look at global transcriptional activation of Wnt/βcatenin pathway we utilized the Wnt reporter construct, pTOP/FLASH, which confirmed that enhanced expression of miR-181a activates this signaling pathway (Fig. 5D).

For the final tasks of this grant we will focus on targeting miR-181a and determine if targeted inhibition of this miRNA can effect tumor growth and rates of recurrence *in vivo*.

Key Research Accomplishments:

- miR-181a is potentially novel prognostic biomarker for advanced-stage ovarian cancer
- miR-181a promotes in vivo dissemination and metastasis
- miR-181a contributes to ovarian cancer tumor initiation through the maintenance of cancer stem cells and targeted inhibition of this miRNA decrease cancer stem cell frequency.
- miR-181a regulates Wnt signaling to promote chemotherapy resistance

Reportable outcomes:

Manuscripts:

1. Nagaraj A, Joseph PL, DiFeo A miRNAs as Prognostic and Therapeutic Tools in Epithelial Ovarian Cancer. Biomark Med. 2015 Mar;9(3):241-57.
2. Parikh A, Lee C, Joseph P, Marchini S, Baccarini A, Kolev V, Fruscio R, Shah H, Mullokandov, Fishman D, Romualdi C, D'Incalci M, Rahaman J, Kalir T, Redline RW, Brown BD, Narla G, and **DiFeo A.** miR-181a induces TGF-β-mediated epithelial-to-mesenchymal transition and promotes epithelial ovarian cancer progression. *Nat Commun*. 2014;5:2977. doi: 10.1038/ncomms3977.
3. Nagaraj A, Joseph PL, Kovalenko O, Singh S, Resnick K, Zanotti K, Waggoner S, **DiFeo A** Wnt/β-catenin signaling regulates platinum resistance in ovarian cancer. Oncotarget. 2015 Jun 29.
4. Enrica C, Paracchini L, Fruscio R, **DiFeo A**, Ravaggi A, Clivio L, Katsaros D, D'incalci M, Marchini S, Romualdi C Integrated analysis to identify regulatory networks associated to patients survival with stage I epithelial ovarian cancer *JCO* (under review)

Employment:

1) Recently received an Impact Score of 15 which was in the 1st percentile on an R01 application based on preliminary data obtained from the grant.

2) Promoted to a tenure-tracked Assistant Professor at the Case Comprehensive Cancer Center, Case Western Reserve University, Cleveland, OH

3) Received Norma C. and Albert I. Geller Designated Professor In Ovarian Cancer Research

Conclusion

Epithelial ovarian cancer (EOC) is the most lethal gynecologic malignancy. Platinum and taxane-based drugs are used in combination as first-line chemotherapeutics for women newly diagnosed with EOC, unfortunately approximately 20% do not respond to this treatment and have very poor outcomes due to chemoresistance, making advanced ovarian cancer particularly difficult to eradicate. The overarching goal of this project is to identify miRNAs involved in the development of ovarian cancer chemoresistance and ascertain their biological relevance using both *in vitro* and *in vivo* models of ovarian adenocarcinoma. In the 5 months that we had this grant, prior to transferring to another institution we were able to complete Specific Aim 1 and uncover novel miRNAs involved in ovarian cancer chemotherapy resistance. Specifically, we found that both the moderately resistant and resistant ovarian cancer cells have similar miRNA profiles. Additionally, we validated several of the dysregulated miRNAs found through the microarray platform and confirmed that the expression of miR-203, -183, -23b, -132 and miR-181a directly correlate with platinum sensitivity. Lastly, through the analysis of a well-annotated expanded clinical cohort of advanced-stage ovarian cancer, we found that high expression of miR-181a correlates with decreased patient survival and this miRNA is enriched in platinum-resistant recurrent tumors, suggesting that it may be a potential prognostic biomarker and therapeutic target for advanced stage ovarian cancer.

Although numerous miRNA profiling studies including our own have uncovered miRNAs that correlate with ovarian cancer patient outcome[1-5] or platinum resistance[6-8] and suggest a link between miRNA signature and chemoresistance, the discrepancy between reported resistance-associated miRNA signatures, the lack of biological validation and functional targets represents major hurdles in understanding miRNAs role in modulating ovarian cancer chemotherapy response. In Specific Aim 2 we set-out to functionally validate miRNAs that show a correlation with patient outcome using both gain- and loss-of-function experiments in a panel of ovarian cancer cell lines with varying sensitivity to platinum therapy. We have uncovered that miR-181a effects numerous cancer-relevant phenotypes such as invasion, metastasis, and increased tumor burden. Lastly, in Specific Aim #3 we will access whether miR-181a can be used as a therapeutic target using either miRNA mimic or antigomers.

Our study, if successful, will identify key miRNAs involved in the regulation of chemoresistance in EOC and systemically validate their biology and function in relevant cell culture and in vivo models of the disease. Targeting these miRNAs using lentiviral based sponge vectors may provide a new paradigm for overcoming chemotherapy resistance clinically and thus improve survival in late stage disease.

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