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TITLE: Circulating Tumor Cell-Based Patient-Derived Xenograft Models of Metastatic Bladder Cancer as a Platform for Development of Novel Therapeutic Approaches

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14. ABSTRACT

A lack of progress in the treatment of advanced bladder cancer stems from, at least in part, from a lack of model systems which recapitulate the evolution of the disease in the context of treatment. In order to address this limitation, we have developed a cohort of patient-derived xenograft (PDX) models derived from circulating tumor cells (CTC) obtained from the peripheral blood of patients with metastatic bladder cancer. Importantly, we have developed paired models from patients derived from CTC samples obtained prior to initiating, and at the time of disease progression on, cisplatin-based chemotherapy. We have performed RNA sequencing of these models to probe mechanisms of cisplatin-resistance identifying multiple pathways associated with cisplatin-resistance. Current work is focused on functional validation of these pathways in an attempt to identify novel therapeutic strategies to prevent/overcome cisplatin-resistance.

15. SUBJECT TERMS
Bladder cancer, Bladder neoplasm, Urothelial cancer, Urothelial neoplasm, cisplatin, chemotherapy, resistance, animal models, DNA damage response

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TABLE OF CONTENTS

INTRODUCTION:

Cisplatin-based chemotherapy represents the mainstay of first-line treatment for advanced bladder cancer with some degree of tumor regression occurring in approximately 50% of patients. However, the vast majority of patients experience disease during, or shortly after, cisplatin-based chemotherapy and only one new drug class (i.e., immune checkpoint blockade) has been approved by the US Food and Drug Administration in this setting in the past several decades. This lack of progress is largely a result of a poor understanding of the pathogenesis of the advanced bladder cancer stemming, at least in part, from a lack of model systems which recapitulate the evolution of the disease in the context of treatment. In order to address this limitation, we have developed a cohort of patient-derived xenograft (PDX) models derived from circulating tumor cells (CTC) obtained from the peripheral blood of patients with metastatic bladder cancer. Importantly, we have developed paired models from patients derived from CTC samples obtained prior to initiating, and at the time of disease progression on, cisplatin-based chemotherapy. These models offer an unparalleled opportunity to study mechanisms of acquired resistance. Genomic profiling of these paired cisplatin-sensitive and -resistant CTC-PDX models has the potential to identify novel potentially targetable pathways driving disease progression.

Hypothesis/Objectives: We hypothesize that CTC-PDX models of advanced bladder cancer can be used to identify targetable mechanisms of cisplatin-resistance. The objectives of this proposal are to expand and molecularly profile this innovative model system platform, characterize the DNA damage response mechanisms that contribute to cisplatin-resistance, and identify novel therapeutic approaches. Specific Aims: In Aim 1 we will (a) expand the cohort of bladder CTC-PDX models and (b) define their genomic alterations. In Aim 2 we will define the DNA damage pathways altered in cisplatin-resistant CTC PDX

1. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Bladder cancer, Bladder neoplasm, Urothelial cancer, Urothelial neoplasm, cisplatin, chemotherapy, resistance, animal models, DNA damage response.

2. **ACCOMPLISHMENTS:**

What were the major goals of the project?

The major goals of the project during year 1 are outlined in the original statement of work below:

	Timeline	**Status**
Aim 1: To (a) expand the cohort of bladder CTC-PDX models and (b) define their genomic alterations		
Major Task 1	Months	
Subtask 1: Amend current IRB and IACUC approvals	1-3	Completed
Subtask 2: HRPO/ACURO Approval	3	Completed

Milestone(s) Achieved		
Local IRB/IACUC Approval	1-3	Completed
Milestone Achieved: HRPO/ACURO Approval	3	Completed
Major Task 2		
Subtask 1: Expand CTC-PDX cohort of cisplatin-sensitive and –resistant models. A total of 110 mice (55 NSG mice for each condition) will be used to generate CTC-PDX models from 25 implanted human bladder CTC samples.	3-12	In process
Milestone(s) Achieved: Establish proof of concept that human advanced bladder cancer experimental models can be generated from bladder CTCs.	12	In process – delayed due to change of institutions of Dr. Domingo-Domenech and need for new regulatory approvals and subcontracts as described below
Major Task 3		
Subtask 1: Complete transcriptional profiling and whole exome sequencing of CTC-PDX models. Genome-wide transcriptome profiling using RNA seq and DNA seq methodology will be used to characterize generated CTC-PDX models.	1-12	RNA sequencing completing and DNA sequencing pending
Subtask 2: Bioinformatics analyses which include GSEA analysis and causal network analysis will be performed in CTC-PDXs transcriptomic profiles to characterize the bladder cancer molecular subtype and identify activated signaling pathways.	8-12	Completed and example of work detailed below
Milestone(s) Achieved: Demonstrate that the CTC-PDX models molecularly recapitulate human bladder cancer subtypes and may facilitate future studies exploring sensitivity to therapies. Catalogue CTC-PDX models to facilitate future studies exploring sensitivity and resistance to therapies directed at recurrent somatic alterations (e.g., FGFR3 mutations). Generate valuable molecular insights to be further explored in Aim 2.	12	Completed pending DNA sequencing
Aim 2: To functionally dissect the DNA damage repair pathways altered in cisplatin-resistant CTC-PDX models		
Major Task 4		
Subtask 1: Functionally validate the list of representative genes upregulated in our patient models of cisplatin resistance assessing their protein expression level by western blot and immunofluorescence analysis in pre- and post-treatment conditions. Western blot and immunoflourescence	1-8	Started with validation of partial list of genes thus far by western blot or IF

studies will be performed in CTC-PDX cells and tissues respectively.		
Subtask 2: Perform a customized siRNA screen to knockdown the expression of the leading edge genes upregulated in the GSEA analysis to determine their contribution to the acquisition of cisplatin resistance. The effect of the siRNA alone or with cisplatin will be measured by MTT and colony formation on CTC-Organoid models and human bladder cancer cell lines (J82 ATCC® HTB-1™ and HT-1197 ATCC® CRL-1473™).	8-16	Started with example for MYC provided below
Subtask 3: Assess activation status of DNA damage and repair proteins kinases in pre- and post-cisplatin treated models by western blot in tissues and CTC-organoid models.	8-16	Not yet started
Milestone(s) Achieved: Characterize the contribution of DNA repair mechanisms to cisplatin resistance in advanced bladder tumors.	16	Partially completed
Aim 3: Determine the pre-clinical anti-cancer effects of targeting DNA damage repair mechanisms in paired cisplatin-sensitive and -resistant CTC-PDX models		
Major Task 5		
Subtask 1: Determine the effect of DNA damage repair inhibitors +/- cisplatin on the number of organoids, cell viability, and induction of apoptosis. Small molecule compounds that inhibit specific DNA damage repair mechanisms will be tested on CTC-Organoids alone or in combination with cisplatin. Apoptosis will be measured through Annexin V and PARP cleavage.	1-8	Not yet started
Subtask 2: Functionally validate the association between the efficacy of a given DNA repair inhibitor and a potential predictive biomarker of response by performing rescue experiments by over expressing DNA repair protein of choice in CTC-organoid models and human cell lines (J82 ATCC® HTB-1™ and HT-1197 ATCC® CRL-1473™).	8-16	Not yet started
Subtask 3: Determine the *in vivo* anti-tumor activity of DNA repair inhibitor of choice in CTC-PDX models. A total of 60 mice harboring CTC-PDX generated from Aim 1/ Major task 2 1will be used to test the activity of DNA inhibitors.	8-24	Not yet started
Milestone(s) Achieved: Identify novel approaches to mitigate cisplatin-resistance in advanced bladder cancer and identify predictive markers of response to DNA damage repair inhibitors.	24	Not yet started
Major Task 6		

Data analysis and manuscript preparation	12-24	Not yet started
Milestone(s) Achieved: Manuscript submission	24	Not yet started

Unfortunately, ~4 months after the award date, Co-PI Josep Domingo-Domenech, an integral team member on this project, was recruited from Mount Sinai to Thomas Jefferson University which has had substantial implications regarding the regulatory and contracting aspects of the project as outlined in detail below. Despite this, we have continued to advance our scientific understanding of our CTC-PDX bladder cancer models in both work, and work not funded, by this mechanism.

What was accomplished under these goals?

Regulatory/Administrative Accomplishments

A timeline of progress to date related to Major Task 1 is detailed below highlighting the impact of Dr. Domingo-Domenech's departure and change of institutions.

1. Mount Sinai IACUC approval 4/5/2017
2. Mount Sinai IRB approval date 7/18/2017 (note, we had initially amended our existing database/biorepository to add the current project on 4/10/2017 – this approach was not felt to be suitable per DoD and a subsequently an independent IRB submission was made)
3. Award notice received 7/19/2018
4. Award effective 8/2017
5. HRPO approval 8/11/2017
6. ACURO approval 9/7/2017
7. Dr. Domingo-Domenech left Mount Sinai to join faculty at Thomas Jefferson 12/2/2017
8. Communication with DoD, Dr. Domingo-Domenech, and administration at Thomas Jefferson regarding modification of SOW and subcontract 12/2017-2/2018
9. Approval of subcontract by DoD 5/22/2018
10. Thomas Jefferson IACUC approval 3/28/2018
11. Mount Sinai IRB continuing review and amendment to include Thomas Jefferson 6/22/18

Scientific Accomplishments

(a) Scientific progress directly related to the current project

Several results with impact for the progress of the project have been generated during this year. Most notably, RNA sequencing of paired CTC-PDX tumors from individual patients obtained prior to, and at the time of progression, cisplatin-based chemotherapy were utilized to generate a "bladder cancer cisplatin progression gene signature". Interrogation of this signature through computational approaches including Gene Set Enrichment Analysis has identified several pathways that are enriched in bladder tumors that have progressed on cisplatin-based chemotherapy as highlighted in the Table below.

Table. Gene sets upregulated in cisplatin-resistant versus cisplatin-naïve tumors

Gene set	NES	p	FDR
E2F_TARGETS	2.68	0.000	0.000
MYC_TARGETS_V1	2.51	0.000	0.000
G2M_CHECKPOINT	1.87	0.000	0.004
MYC_TARGETS_V2	1.84	0.002	0.004
DNA_REPAIR	1.71	0.000	0.010
OXIDATIVE_PHOSPHORYLATION	1.70	0.000	0.009
SPERMATOGENESIS	1.58	0.014	0.021
REACTIVE_OXIGEN_SPECIES_PATHWAY	1.41	0.052	0.070
UNFOLDED_PROTEIN_RESPONSE	1.40	0.025	0.068
INTERFERON_ALPHA_RESPONSE	1.33	0.066	0.104

NES, Normalized enrichment score; FDR, false discovery rate

Intriguingly, gene set enrichment analysis (GSEA) of differentially expressed genes have revealed that our bladder cancer cisplatin progression gene signature is significantly enriched in the DNA repair gene expression as well as MYC signaling genes (**Figure 1A**). We have further confirmed these results by performing immunohistochemical studies on the paired tumor samples demonstrating an increase of MYC protein expression levels in tumors progressing on cisplatin (**Figure 1B**). Furthermore, MYC rise was paralleled by an increase in cancer cell proliferation, measured via immunohistochemistry for Ki67 (**Figure 1C**).

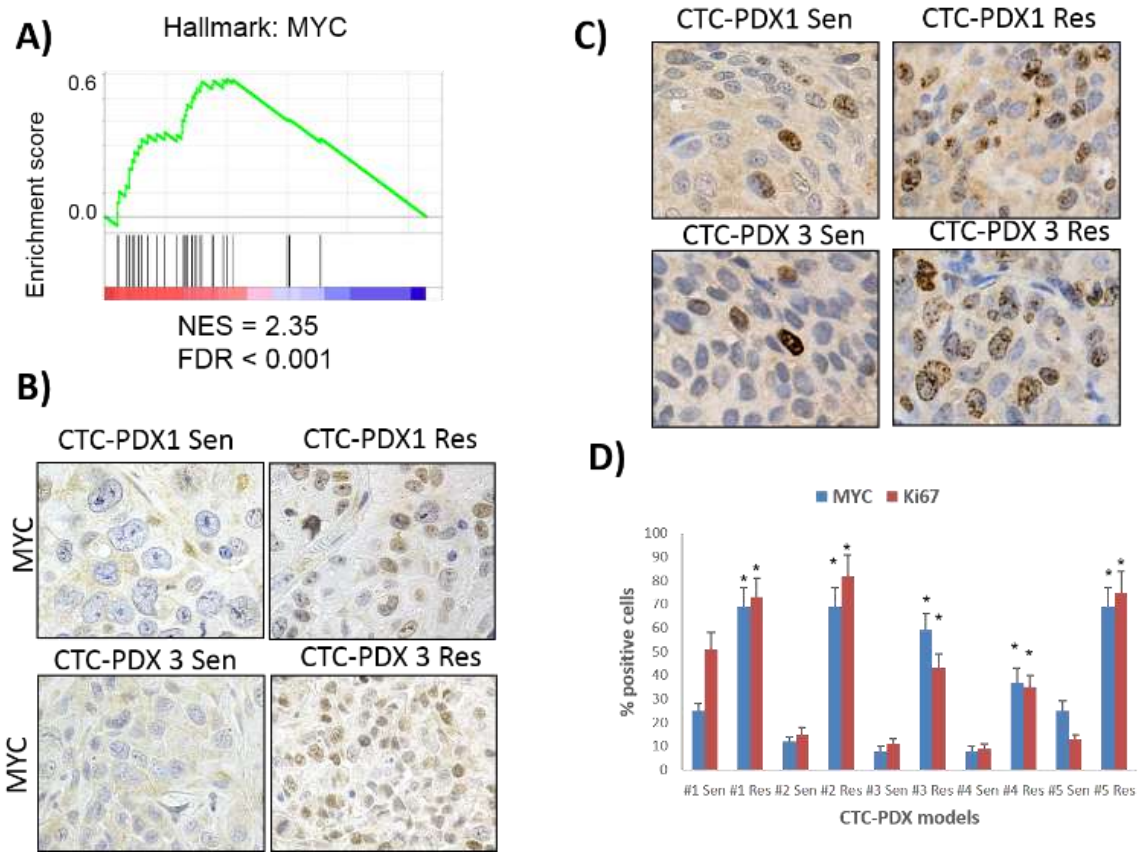

Figure 1. MYC pathway activity increases in bladder cancer tumors that progress to cisplatin. A) GSEA score curves. Enrichment plots are shown for MYC pathway in positively correlated genes in cisplatin resistant bladder CTC-PDX models. Representative immunohistochemistry of B) MYC and C) Ki67 in paired sensitive and resistant PDX models. D) Quantification of positive cells from B) and C). Bars represent mean ± SD. *p≤0.05. Bar = 100μm.

We subsequently performed functional genetic studies using silencing RNAs against MYC in bladder cancer cells and showed that although MYC knockdown impacted on the size of colonies, it did neither sensitize cells to cisplatin (**Figure 2A**) nor increase apoptotic cell death (**Figure 2B**). Collectively, these results indicate the complexity of MYC pathway signaling in bladder cancer progressing despite cisplatin-based chemotherapy and suggest that an enrichment of MYC pathway genes in the cisplatin-resistant gene signature may reflect the proliferative state of platinum-resistant bladder cancer cells but not be directly related to resistance to platinum-therapy. Further studies are being pursued to understand better the contribution of MYC to the pathogenesis of bladder cancer and identify novel targetable bladder cancer vulnerabilities.

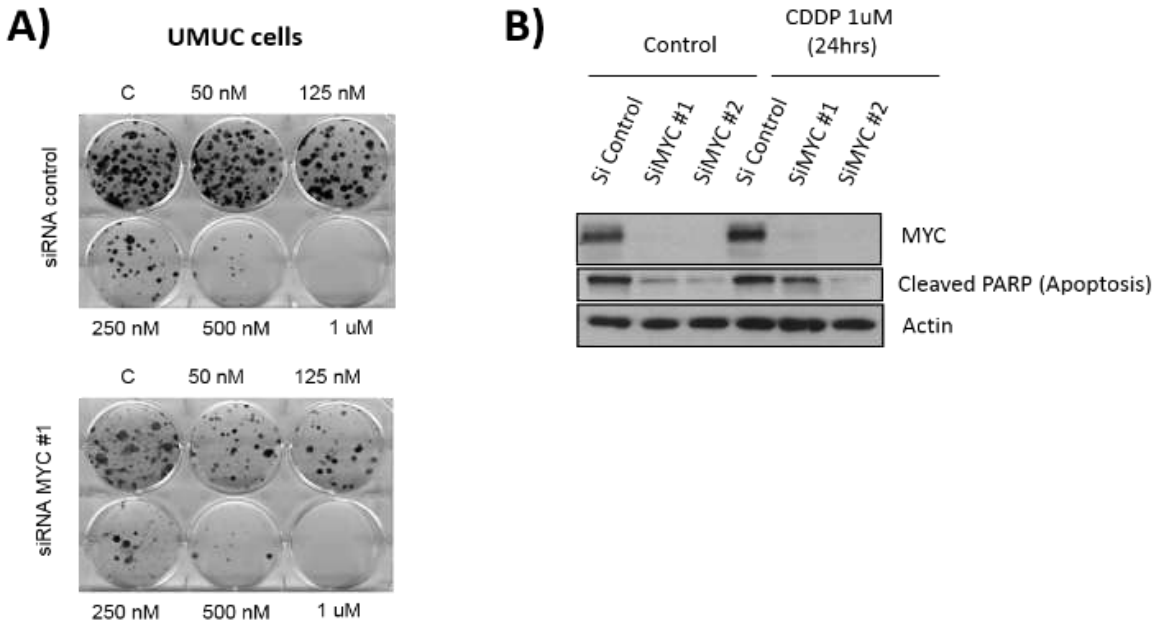

Figure 2. MYC depletion does not enhance cisplatin resistance. A) Colony formation assays of UMUC cells transfected with control and MYC siRNAs and treated with CDDP for 72 hours. B) Immunoblot of MYC and cleaved PARP of same cells as (A).

(b) Scientific progress indirectly related to the current project

We, and others, have demonstrated a positive correlation between T-cell infiltration and epithelial-mesenchymal transition (EMT)-related gene expression in bladder cancer. Given that T-cell infiltration is generally associated with a favorable prognosis in bladder cancer whereas the biological process of EMT has been linked to treatment-resistance and poor prognosis, this finding raised several questions principally among which were: What was is the cellular origin of EMT-related gene expression in bulk transcriptomes? Does EMT-related gene expression actually represent the biological process of EMT? To help address these questions, we leveraged our set of bladder cancer CTC-PDX models given that the transcriptome in these models is a mixture of human RNA (derived from cancer cells) and mouse RNA (derived from stromal cells). We analyzed RNA sequencing (RNAseq) data from five UC PDX models and used the Bamcmp algorithm34 to separate RNAseq reads derived from mouse versus human and demonstrated that the EMT-related gene signature largely emanates from stroma rather than cancer cells and likely is a reflection of the stromal contribution of the tumor rather than a reflection of the biological process of EMT. This work is currently in press and further highlights the utility of our models for generating insights relevant to the biology of human bladder cancer (Wang et al, *Nature Communications*, In Press). Though this work is distinct from the work proposed in the current project, we believe that is reinforces our ability to utilize our models to advance the science of bladder cancer and we expect similar progress on the current project once the subcontracting is finalized.

What opportunities for training and professional development has the project provided?

The project has provided the opportunity for one-on-one work between mentor, Josep Domingo-Domenech and mentee, Jung Reem Wood.

How were the results disseminated to communities of interest?

Nothing to report.

4. IMPACT:

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

To date, this project demonstrates that the development of xenografts from patient-derived circulating tumors cells is feasible and may lead to insights regarding treatment resistance in human bladder cancer. The potential advantages of circulating tumor cell-based models, versus conventional organ-based patient-derived xenograft models, are several fold including: (a) the ability to interrogate tumor specimens serially from the same individual in the context of treatment sensitivity and resistance and (b) the ability to interrogate cancer cell populations that might represent the most lethal clones (i.e., disseminating cells).

What was the impact on other disciplines?

The development of CTC-PDX models is not limited to bladder cancer and may be extended to multiple other tumor types. Indeed, we have utilized prostate cancer CTC-PDX models to generate insights regarding resistance to treatment in metastatic castration-resistant prostate cancer (Vidal et al, Cancer Cell, 2015).

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Though there have not been changes in the approach, the SOW has been modified (after discussion with the Scientific Officer) based on the change of institution of Dr. Domingo-Domenech.

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

There have been delays to the project based on the change of institutions of Dr. Domingo-Domenech and as outlined in detail in Section 2. Unfortunately, this change occurred very

shortly after regulatory approvals had initially been approved resulting in another set of delays amending the Mount Sinai regulatory documents to encompass Thomas Jefferson, establishing a subcontract, and submitted the project for regulatory approval at Thomas Jefferson. The process is in the final stages of completion and the SOW has been revised setting the stage for year 2 of work on this project.

Changes that had a significant impact on expenditures

The departure of Dr. Domingo-Domenech and requirement for a subcontract encompassing the indirect costs has had an impact on the initial budget and is reflected in the revised budget.

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

Nothing to report

Significant changes in use or care of human subjects

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

6. PRODUCTS:

- **Publications, conference papers, and presentations**

  Nothing to report

  Journal publications

  Nothing to report

  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

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Matthew D. Galsky, MD
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	0000-0001-7655-937
Nearest person month worked:	2.4
Contribution to Project:	Dr. Galsky has overseen all aspects of the project including coordinating regulatory approvals, collection of peripheral blood specimens for CTC-derived PDX models from patients with metastatic bladder cancer, and generation and interpretation of data.

Name:	Josep Domingo-Domenech, MD PhD
Project Role:	Co-PI
Researcher Identifier (e.g. ORCID ID):	0000-0002-7870-7511
Nearest person month worked:	2.4
Contribution to Project:	Dr. Domenech laboratory has generated and maintained the CTC-derived PDX models and organoids utilized in the current project and has performed experiments to functionally validate pathways identified as putative drivers of cisplatin-resistance.

Name:	Veronica Rodriguez-Bravo, PhD
Project Role:	Co-I
Researcher Identifier (e.g. ORCID ID):	0000-0002-7363-3672
Nearest person month worked:	0.6
Contribution to Project:	Dr. Rodriguez-Bravo has analyzed DNA repair pathways in the bladder CTC-PDX models and is assisting in delineating the experiments necessary to interrogate the contribution of these pathways to cisplatin progression in bladder cancer.

Name:	Yujin Hoshida, MD PhD
Project Role:	Co-I
Researcher Identifier (e.g. ORCID ID):	Not available
Nearest person month worked:	0.6
Contribution to Project:	Dr. Hoshida has performed computational analysis of RNA sequencing data from the CTC-PDX models including generation of a cisplatin-resistance signature and GSEA to identify putative pathways associated with resistance.

Name:	Manpreet Brar
Project Role:	Research Coordinator
Researcher Identifier (e.g. ORCID ID):	Not available
Nearest person month worked:	4.8
Contribution to Project:	Ms. Brar is a research coordinator who has been responsible for organizing and submitting regulatory documents to local ethics committees. She is also coordinating collection of peripheral blood specimens from the outpatient genitourinary oncology clinic.

Name:	Jung Reem Woo, PhD
Project Role:	Post-Doctoral Fellow
Researcher Identifier (e.g. ORCID ID):	Not available
Nearest person month worked:	4.8
Contribution to Project:	Dr. Woo, under the mentorship of Dr. Domingo-Domenech, has been responsible of generating the CTC-PDX and organoid models. She has also performed the studies focused on dissecting the mechanisms that contribute to cisplatin resistance.

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?

Organization Name: Thomas Jefferson University
Location of Organization: Philadelphia
Partner's contribution to the project (identify one or more)

- Facilities
- Collaboration

8. SPECIAL REPORTING REQUIREMENTS

Nothing to report

9. APPENDICES:

Nothing to report