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TITLE: Mechanisms of Action and Resistance to CDK4/6 Inhibitors in Breast Cancer

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14. ABSTRACT

CDK4/6 inhibitors combined with endocrine therapy (ET) are mainstay to treat metastatic estrogen receptor (ER) positive patients. Yet, almost 60% develop resistance to CDK4/6 inhibition within 2 years of initial treatment. An ongoing clinical challenge has thus been identifying biomarkers of response to predict patients that will either respond or not respond to palbociclib. Further, there is an unmet need to identify actionable targets for patients that have progressed on CDK4/6 blockade regimens. Currently, the only biomarker being used to identify patients for anti-CDK4/6 therapy is estrogen receptor (ER) by IHC. The goal of my study was thus to identify the therapeutic vulnerabilities of CDK4/6 inhibitor resistance cells and identify key markers that can longitudinally correlate with development of resistance. My data suggests that resistance to palbociclib results in a cascade of events initiating with induction of autophagy and senescence, leading to the promotion of senescence-associated secretory phenotype (SASP) that affects surrounding cells and promotes tumor growth.

15. SUBJECT TERMS

Breast cancer, estrogen receptor-positive, CDK4/6, cell cycle, drug resistance, autophagy, senescence, STAT3, IL-6, FOXO3, Beclin-1, patient-derived xenograft, disease progression, PARP inhibitor, TTI-101, SASP

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INTRODUCTION:

The proposed research aims to lead to a solution for the following overarching challenges: (1) Revolutionize treatment regimens by replacing them with ones that are more effective, less toxic, and impact survival. (2) Conquer the problems of over-diagnosis and overtreatment. (3) Eliminate the mortality associated with metastatic breast cancer. Various studies have tried to understand the mechanism of the CDK4/6 inhibitors, but no study to date examined the dose dependent effect of the inhibitor. I proposed that inhibition of autophagy could allow lower doses of palbociclib, which could augment its overall benefit with little or no added toxicity and at low cost. In addition, the impact of the approach of the experiments proposed could inform additional therapeutic strategies and improve personalization of therapy. Moreover, this proposal will elucidate the dose-dependent mechanisms of the CDK4/6 inhibitors and will identify proteins in the autophagy pathway that are direct targets of CDK4/6. Lastly, close examination of the mechanisms of palbociclib resistance to identify biomarkers of resistance and treatment strategies to circumvent the acquired resistance.

KEYWORDS: Breast cancer, estrogen receptor-positive, CDK4/6, cell cycle, drug resistance, autophagy, senescence, STAT3, IL-6, FOXO3, Beclin-1, patient-derived xenograft, disease progression, PARP inhibitor, TTI-101, SASP

ACCOMPLISHMENTS:

What were the major goals of the project & what was accomplished under these goals?

The following aims and tasks that have been addressed to reach the goals of the project:

Specific Aim 1: Identify the molecular mechanism by which CDK4/6 regulates autophagy pathway

In progress (60% effort year 2; 25% effort year 1)

✓ Major Task 1: Generate CDK4, CDK6, CDK4-KD, and CDK6-KD protein expression vectors

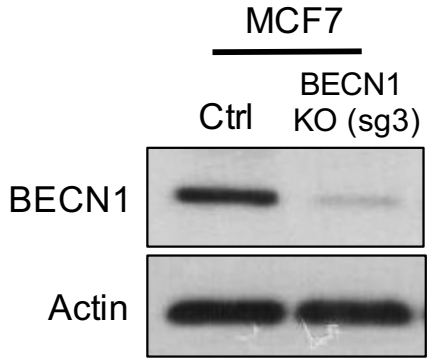
CDK4 & CDK6 protein expression vectors were generated in year 1. In progress is the development of kinase dead (KD) CDK4 and CDK6 cDNA to then clone into the previously generated vectors reported in year 1. The following primers were used to mutate CDK4 and CDK6:

CDK4 threonine 172 to alanine primer 1: 5'-ATGGCCCTCGCGCCTGTGGTG-3'
CDK4 threonine 172 to alanine primer 2: 5'-CACCACAGGCGCGAGGGCCAT-3'
CDK6 threonine 177 to alanine primer 1: 5′ -CACCATGGAGAAGGACGGCCTG-3′
CDK6 threonine 177 to alanine primer 2: 5′ -GCAGAGCCTGTCCAGAAGAC -3′

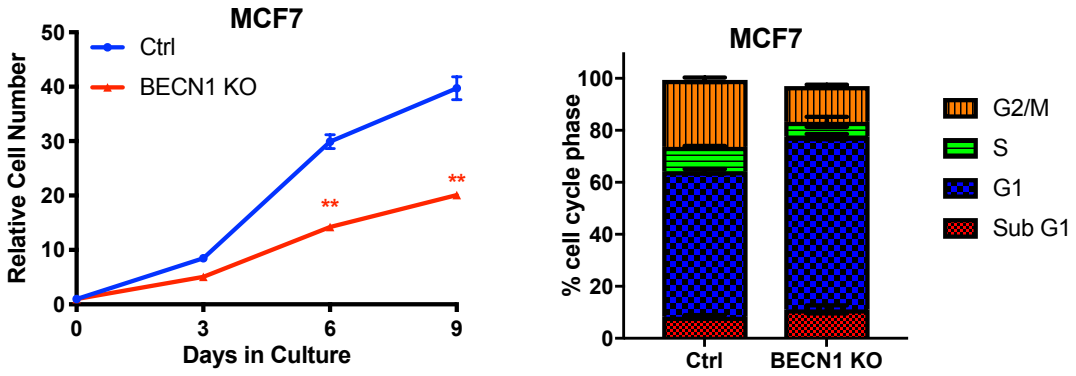
Further validation of the kinase dead vectors is ongoing and will be reported in the next reporting period.

✓ Major Task 2: Evaluate status of Beclin-1 activating and inhibitory complexes *in vitro*

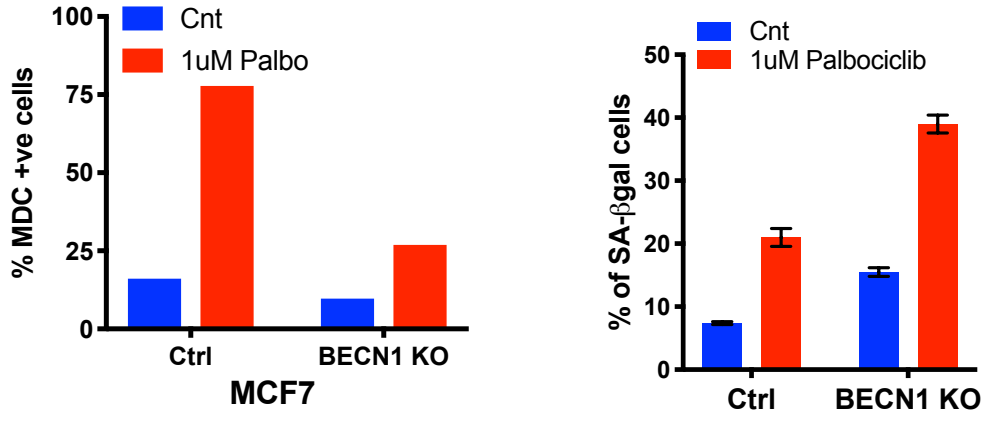
Further downstream analysis of the cells with Beclin-1 knockout generated in year 1 was performed. Knockout (KO) of Beclin-1 (BECN1) was confirmed by protein analysis of whole cell lysates as shown in the immunoblot figure below.



Upon examination of the cell growth rate of the BECN1 KO cells, loss of BECN 1 suppresses the normal growth rate of the cells as shown in the growth curves below. Additionally, this suppression in growth rate is correlated with increased in cell cycle arrest (increase in percent of cells in G1) as shown in the bar graph below.

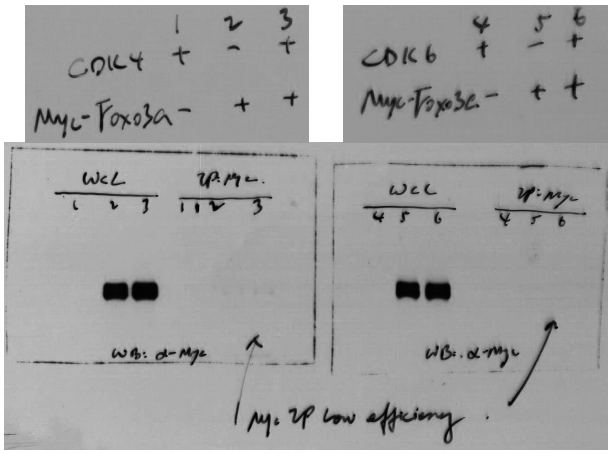


Testing the effect of palbociclib induced autophagy in the Beclin-1 knockout cells, results in decreased autophagy (MDC) and increased senescence (SA-βgal) as shown in the bar graphs below.

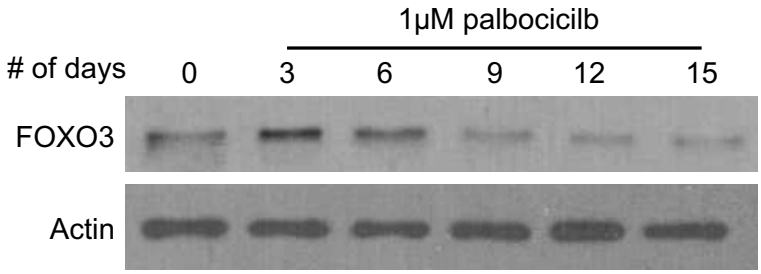


✓ Major Task 3: Identify phosphorylation events by CDK4/6 on autophagy proteins

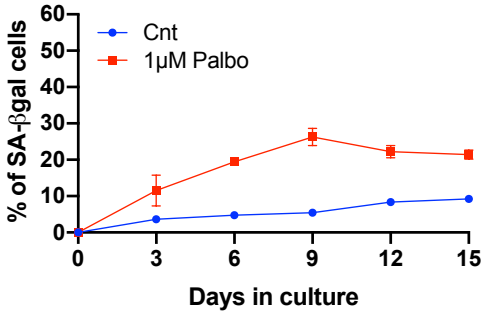
Specific phosphorylation events have yet to be identified, due to low quality of immunoprecipitation (IP) experiments see image of immunoblot below. Troubleshooting of IP experiments is ongoing and will be reported in the next reporting period.



Due to the delay with IP experiments, I started investigating the alternate strategy proposed in my original proposal that the regulation of autophagy by CDK4/6 is through an indirect mechanism such as E2F regulation. Previous studies have shown that FOXO3 is transcriptionally regulated by E2F and is important in induction of autophagy as well as senescence. I have investigated the expression of FOXO3 over time upon palbociclib treatment. FOXO3 is initial induce upon palbociclib treatment, but then levels decrease as observed in the immunoblot below.



This observation may explain the switch overtime that initial treatment with palbociclib induces autophagy but continued treatment leads to senescence. I have also observed that levels of senescence (SA-βgal) peak after 9 days of treatment and then maintain a steady state of senescent cells.



Senescent cells secrete interleukins, inflammatory cytokines, and growth factors, which comprise the senescence-associated secretory phenotype (SASP) that affects surrounding cells and promote tumor growth. The most prominent SASP cytokine is interleukin-6 (IL-6), which links to the mechanisms evaluated in aim 2. Further investigation is ongoing of the regulation of autophagy and senescence by CDK4/6 to determine if regulation of senescence versus autophagy could be a key driver of resistance.

✓ Major Task 4: Evaluate the effects of CDK4/6 phosphorylation on the status of Beclin-1 activating and inhibitory complexes *in vitro*

Experiments have not started, but will begin in next reporting period. Need to validate kinase dead vectors ongoing in major task 1.

Specific Aim 2: Examine the mechanisms of resistance to palbociclib and identify treatment strategies to circumvent such resistance

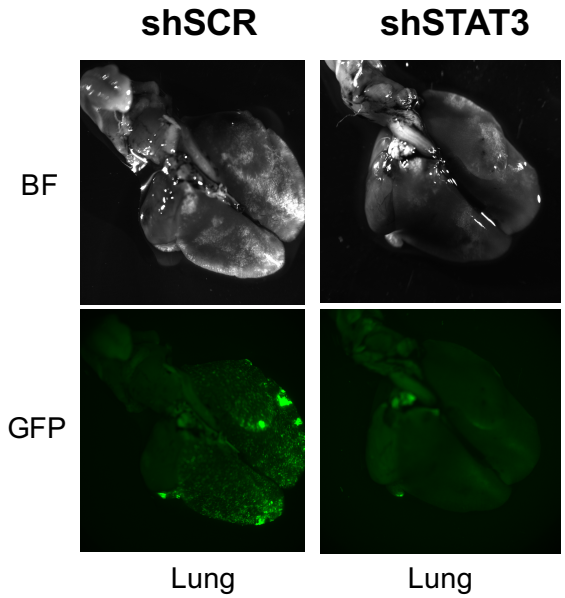
In progress (20% effort year2; 70% effort year 1)

✓ Major Task 5: Evaluate the role of IL-6/STAT3 pathway in palbociclib resistance cells

Completed subtask 1 & 2 in year 1 and findings were reported in year 1 progress report.

Subtask 3: Implantation of stable cell lines produced in subtask 2 via tail vein injection in mice and monitor tumor growth, metastasis, and survival.

I have tested whether the knockdown of STAT3 in the resistant cells hinder the metastatic potential. I have tail vein injected 20 mice (5 mice/group) with either MCF7 or T47D shSCR and shSTAT3#2 (chosen since it is observed to be the best knockdown as reported in year 1). After 4 months, lungs were isolated and were imaged for GFP since the cells has reported in year 1 express GFP. Knockdown of STAT3 seems to hinder metastatic lesions in the lung as shown by representative images below.



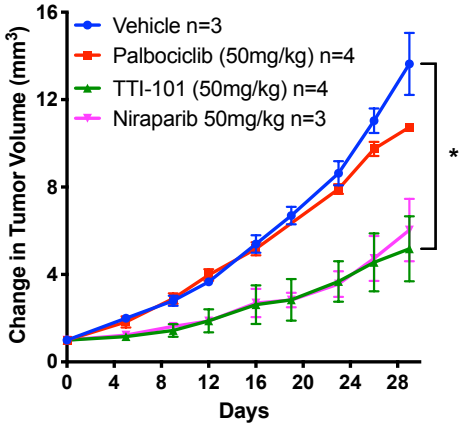
✓ Major Task 6: Examine the effect of IL-6/STAT3 inhibitors in combination with drugs targeting DNA damage *in vitro* and *in vivo*

Completed subtask 1 in year 1 and findings were reported in year 1 progress report.

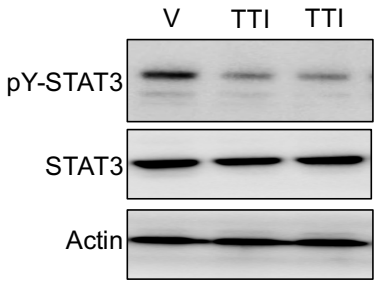
Subtask 2: Based off results in subtask 1, I will use the most synergistic combination to carry out implantation of palbo resistant cells in mice and monitor tumor growth, metastasis, and survival

Due to the development of tumors from the cell line xenograft in the mammary fat pad being very slow growing as discussed in year 1 report, I have collaborated with Dr. Meric-Bernstam to obtain patient-derived xenografts (PDX) to test the treatment strategies as discussed as an alternative in the original proposal. I obtained 2 models: model 1 is a PDX from a patient who was on palbociclib + endocrine therapy for greater than 6 months prior to progression & model 2 is from a patient who was on therapy for less than 6 months prior to progression. I have tested the effect of STAT3 inhibitor (TTI-101) in both models. Below are the graphs representing the change in tumor volume with and without treatment. Model 1 shows a significant delay in tumor growth upon treatment with TTI-101, but not model 2. I confirmed in Model 1 the inhibition of activate STAT3 (decreased pY-STAT3) by protein levels by TTI-101. Ongoing analysis is being conducted to determine the difference in response between to the two models. Additionally, models have been obtained from Dr. Meric-Bernstam to validate the therapeutic benefits.

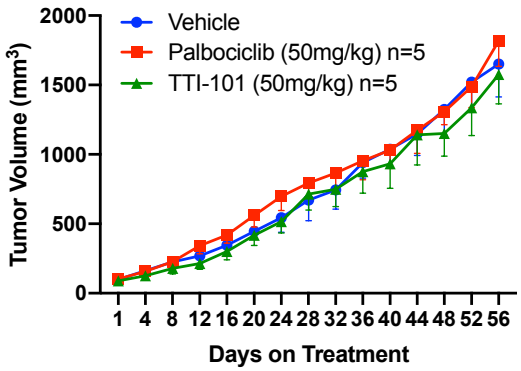
Model 1



Model 1



Model 2



Specific Aim 3: Classify clinically applicable biomarkers to identify patients with intrinsic or acquired resistance to palbociclib.

In progress (20% effort year 2; 15% effort year 1)

✓ Major Task 7: Optimize antibody conditions of candidate biomarkers of response or resistance identified in Aim 1 & 2

Antibody conditions are being optimized for γH2AX as a potential biomarker of resistance. pY-STAT3 staining conditions are optimized, however ongoing efforts in collaboration with Dr. Sahin are being conducted to optimize the scoring of the stain. Additional biomarkers are being identified as the experiments in Aim 1 are being completed. Potential candidates in autophagy and SASP are being investigated in vitro and will be tested for immunohistochemistry in the next reporting period.

✓ Major Task 8: Validate optimized antibody conditions in cohort of breast cancer patients

Not started, as planned in SOW. I have a panel of breast cancer tissue (1600 FFPE blocks), not the palbociclib treated cohort as in major task 7 & 9, to determine the reliability of the antibodies tested.

✓ Major Task 9: Obtain breast cancer patients samples whose disease has progressed after palbociclib treatment

In progress. I have been collecting FFPE blocks/slides from the retrospective data base to obtain patient samples treated with palbociclib and obtain wide-range of patients from responders, non-responders, and recurrent. Below is the current FFPE block/slides obtained.

Overall accomplishment summary

My main accomplishment for this reporting period is the results reported in Aim 2 indicating that TT1-101 is safely tolerated and efficacious at the dose tested and that TTI-101 may be a suitable target for those tumors that are resistant to palbociclib. There were differences in response to TTI-101 between early progression (intrinsic) and late progression (acquired) on CDK4/6 inhibitor suggesting that IL-6-STAT3 signaling axis may be a driver in acquired palbociclib resistance. Our ongoing studies are therefore geared towards testing the efficacy IL-6-STAT3 inhibition in both settings and identifying distinct therapeutic vulnerabilities of intrinsic palbociclib resistance. Additionally, the findings discovered in aim 1 during this reporting period are leading to additional biomarker discovery that will be further investigated and validate in the next reporting period.

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

Date of Training or Professional Development	Event	Description
3/20/2020- 3/23/2020	Suspension of laboratory research	Organized and led the activities for suspension of research
03/08/2020	Houston Livestock Show & Rodeo Youth Agricultural Science Fair	Served as official judge for Junior division
02/01/2020	MD Anderson Office of Translational Research Thematic SPORE Symposium	Attended symposium on immunotherapy based projects across multiple disease sites
01/31/2020	MD Anderson Breast Cancer Clinical & Translational Research Conference	Invited speaker for "Beyond CDK Inhibitors: Informing New Trial Designs" session and gave oral presentation on "Reversing CDK4/6i Resistance: STAT3 & PARP inhibition"
01/08/2020 - 1/15/2020	Clinical rotation in the Dept. of Pathology at MD Anderson	Shadowed Dr. Aysegul Sahin and her clinical team
12/10/2019 - 12/14/2019	2019 San Antonio Breast Cancer Symposium	Presented two posters: "STAT3 as a therapeutic target in estrogen receptor positive breast cancer patients refractory to CDK4/6 inhibition." & "Role of IL-6 in promoting endocrine therapy and palbociclib resistance estrogen receptor positive breast cancer cells."
12/09/2019	Invited Speaker for Dr. David Tweardy's Laboratory at MD Anderson	Oral Presentation "Investigation of the modes of resistance to CDK4/6 inhibitor therapy and therapeutic strategies"
12/04/2019	Novartis Ribociclib Virtual Medical Education Program	Virtual meeting sharing the latest data from MONALEESA trail program and Q&A session with Novartis biomedical research team
11/14/2019	CPRIT Shark Tank Elevator Pitch Competition	90-second pitch of research in language & relevance for a clinical audience. Ranked above the mean score.
11/01/2019	Committee Meeting	Presentation updating research progress to my collaborators
9/25/2019 & 10/23/2019	CPRIT Shark Tank Elevator Pitch Seminar & Training	Attended seminar about elevator pitches and attended practice training session
10/16/2019 - 2/26/2020	Postdoctoral Association Executive Committee Career Development Vice Chair	Facilitated networking with potential speakers for seminar series

10/14/2019	Electronic Lab Notebook (ELN) Focus Group Future State Assessment	Participated in assessment of the foundation to select and implement an appropriate ELN
8/19/2019 - 8/20/2019	Electronic Lab Notebook (ELN) Focus Group Current State Assessment	Participated in assessment of current lab notebook practices and provide capturing needs by a future ELN
08/16/2019	Experimental Radiation Oncology Elevator Pitch	Presented 2-minute elevator speech at departmental showcase event
08/09/2019	CPRIT CURE Summer Undergraduate Program Poster Competition	Served as scientific judge for competition
06/11/2019 - 08/16/2019	CPRIT CURE Summer Undergraduate Program	Mentor and trained CPRIT CURE Summer undergraduate student

How were the results disseminated to communities of interest?

At the San Antonio Breast Cancer conference, I had two patient advocates approach my poster presentation on "STAT3 as a therapeutic target in estrogen receptor positive breast cancer patients refractory to CDK4/6 inhibition." I discussed with the patient advocates the therapeutic benefit observed in my preclinical mouse models of palbociclib resistance, which is going to be proposed as a clinical trial in the near future.

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

Over the next reporting period I plan to complete the analysis of the molecular mechanisms by which CDK4/6 regulates the autophagy and senescence pathway. This will allow additional biomarkers of sensitivity and resistance to CDK4/6 inhibition to be identified. Upon the identification of the biomarkers, I plan to complete the application of the biomarkers to the retrospective patient samples. The completion of the validation of identified biomarkers in addition to the further validating the therapeutic benefits of the proposed targeted therapies will provide guidance to my collaborators for use in clinical trials.

IMPACT:

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

The precise biological mechanism(s) of the action or resistance to CDK4/6 inhibitors (palbociclib, ribociclib, and abemaciclib) are still unknown and there are no independent biomarkers to predict response and/or resistance to these inhibitors. Patients who experience resistance to this class of agents, are likely to have a less favorable biology (including resistance pathways), requiring novel combination strategies to delay progression and improve survival. Over this past year I confirmed the therapeutic benefit of STAT3 and PARP inhibition in preclinical mouse models of palbociclib resistance. This will provide rationale to propose novel clinical trials to

my collaborators in Breast Medical Oncology and Investigational Cancer Therapeutics at MD Anderson Cancer Center leading to better outcomes for our patients. Additionally, I have identified some key mediators (or drivers) of palbociclib resistance that could be used as biomarkers to predict the development of resistance and allow earlier intervention of next-line therapy improving overall clinical outcomes.

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

CHANGES/PROBLEMS:

Nothing to Report

PRODUCTS:

Journal publications

Nothing to Report

Books or other non-periodical, one-time publications

Nothing to Report

Other publications, conference papers, and presentations

Please refer to table on page #10 which lists all conferences and presentations made during the last year.

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

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:

What individuals have worked on the project?

Name:	Nicole M. Kettner
Project Role:	PI
Researcher Identifier:	ORCID: 0000-0003-2043-4407
Nearest person month worked:	12
Contribution to Project:	Dr. Kettner has performed all the work that has been performed this reporting period
Funding Support:	

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

SPECIAL REPORTING REQUIREMENTS:

COLLABORATIVE AWARDS: Nothing to Report

QUAD CHARTS**:** Nothing to Report

APPENDICES:

Nothing to Report