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PRINCIPAL INVESTIGATOR: Chun-Ju Chang

CONTRACTING ORGANIZATION: Purdue University
West Lafayette, IN 47907-2040

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Chun-Ju Chang, Meng-Ju Wu, Yingsheng Zhang

E-Mail:chunjuchang@purdue.edu

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14. ABSTRACT
Cancer stem cells (CSCs), a cell population with acquired perpetuating self-renewal properties which resemble normal stem cells, specifically in the ability to infinitely give rise to the bulk of a tumor as the "seed" of the cancer, account for cancer initiation, progression, recurrence, and chemo-resistance. The cell polarity machinery has been strongly suspected of playing an evolutionarily-conserved role in regulating the cell fate in both normal and neoplastic stem cell populations, which suggests that therapeutic targeting of this mechanism may be an effective strategy for eliminating CSCs and thereby impeding cancer progression and recurrence. During the first grant period, we have successfully completed the proposed studies in Aim1 and have also made significant progress in the ongoing experiments in Aim2. Our results collectively support the hypothesis that PKCzeta is a novel target of microRNA-200c (miR-200c), the most significantly down-regulated miRNA in breast CSCs. Dysregulation of miR200c-PKCzeta signaling is critical for sustaining a self-renewing breast CSC pool and is associated with high-grade aggressive breast cancer. Together, these data point to a great potential for the strategies targeting PKCzeta signaling to exhaust the CSC pool for treatment of breast cancer. The project was not able to be completed during original grant period because the very large datasets generated from the proposed high throughput compound screening experiment in the major task 5 have taken longer than expected to be analyzed and validated, and a no-cost extension has been approved to change the project expiration date to September 29, 2019 that would allow us to conclude all the proposed work, and have the final data incorporated into a manuscript for publication.

15. SUBJECT TERMS
Breast cancer, PKCzeta, MicroRNA, Cancer stem cell, Cell fate determinant, Cell polarity

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Table of Contents

Page

1. Introduction

Cancer stem cells (CSCs), a cell population with acquired perpetuating self-renewal properties which resemble normal stem cells, specifically in the ability to infinitely give rise to the bulk of a tumor as the "seed" of the cancer, account for cancer initiation, progression, recurrence, and chemo-resistance. To date, treatment strategies designed to eliminate CSCs still remain a significant challenge, and delineation of the underlying mechanism(s) governing the cell fate decision to maintain self-renewal properties in CSCs likely holds the key to the development of effective treatments that can eradicate the genesis of cancer. The cell polarity machinery has been strongly implied to play an evolutionarily-conserved role in regulating cell fate in both normal stem cells and cancer stem cells (CSCs), suggesting that therapeutic targeting of this mechanism may be an effective strategy that can be applied to eliminate CSCs and thereby to impede cancer progression, recurrence, and chemo-resistance. However, the precise critical cell polarity components and mechanisms involved in the regulation of CSC cell fate still remain to be defined. Notably, asymmetric divisions (AD) is a critical mechanism which ensures self-renewal during proliferation of mammalian stem cells, where a family of cell polarity proteins, atypical Protein Kinase C (aPKC), phosphorylates the cell fate determinant NUMB, which in turn directs the polarized distribution of NUMB exclusively to the daughter cell with the differentiated cell fate, allowing the opposite daughter cell that accumulates aPKC to maintain the stem cell identity. In contrast, loss of PKCζ expression/activity leads to a uniform distribution of NUMB with the consequent symmetric commitment (SC) of both daughter cells to the differentiated cell fate, resulting in exhaustion of the stem cell pool. Interestingly, our preliminary data provide the first evidence showing that a member of the aPKC family, PKCζ, is a novel target of microRNA-200c (miR-200c), a microRNA known to be significantly down-regulated in breast CSCs. Our own previous findings and the preliminary results in this study further elucidate that loss of miR-200c not only leads to the gain of stem cell properties to generate a CSC-like population, but also enhances AD to sustain the CSC pool, potentially through upregulation of PKCζ. Even though these findings provide evidence to support a role of miR200c-PKCζ axis in regulation of breast CSCs, the precise underlying mechanism that links the regulation of PKCζ to the breast CSC fate remain to be delineated, and the analysis system to elucidate the dynamic changes of the CSC fate decision (AD vs. SC) has yet to be established. As a consequence, there remains a critical need to determine the mechanisms by which the CSC fate is regulated, since, in the absence of such knowledge, the development of effective therapeutic interventions to target CSCs and prevent cancer progression and recurrence will likely remain limited. Based on supporting evidence and our own preliminary data, our central hypothesis is that upregulation of PKCζ expression is critical for promoting AD to sustain a self-renewing CSC pool, and that strategies targeting PKCζ signaling will be therapeutically effective in treating breast cancer by exhausting CSCs. To test the hypothesis, we propose the following aims: Aim 1 will determine the key cell polarity mechanism(s) involved in regulation of breast CSCs, and Aim 2 will develop a therapeutic strategy targeting the cell polarity machinery to direct breast CSC fate. At the completion of this project, it is our expectation that we will have revealed a novel role of miR-200c- PKCζ signaling in regulation of the polarity of breast CSC division and the consequent cell fate and have provided new and important clinical implication of PKCζ inhibitor in breast cancer treatment. Under the support of this award, we have made the following progress during the **third** grant period (Sep 30, 2017- Oct 1, 2018). We have added

new preliminary data and also revised some of the preliminary data from the previous terms. The finalized data that has been published in Oncogene (2017) Jun 1; 36(22), 3193-3206 is cited with journal reference in the report.

2. Keywords

Breast cancer, PKCzeta, MicroRNA, Cancer stem cell, Cell fate determinant, Cell polarity

3. Accomplishments

- **Goals and Accomplishments:**

Specific Aim 1: Determine the key cell polarity mechanism(s) involved in regulation of breast CSCs

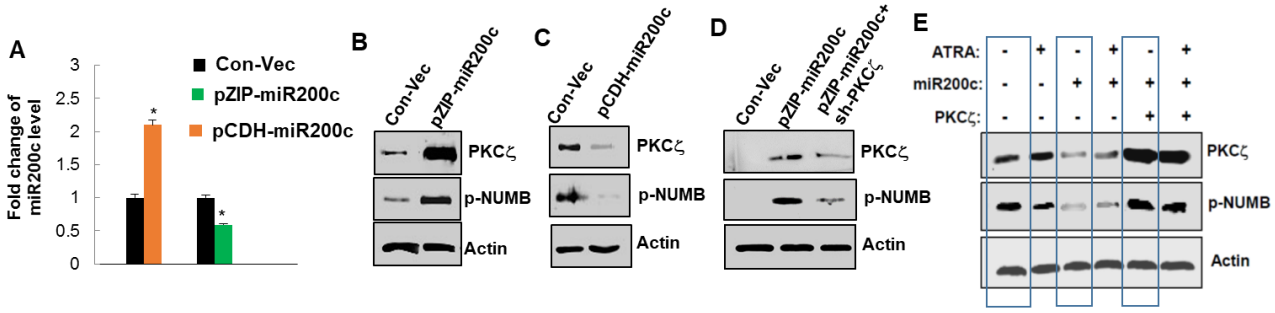


Figure 1. PKCζ is a bona fide miR-200c target. (A) MiR-200c expression levels (n=3 independent experiments, asterisk indicates P<0.05, error bars denote ±SD), and **(B-C)** PKCζ and p-NUMB protein levels in T47D cells that stable expressed pZIP-miR200c and pCDH-miR200c, and in the control cells expressing the control vectors. **(D-E)** PKCζ and p-NUMB protein levels in MCF7-pZIP-miR200c cells that further expressed sh-PKCζ, or in pCDH-miR200c expressing TNBC cells that further expressed PKCζ cDNA or the control vectors.

Major Task 1: Determine the role of miR200c-PKCζ signaling in regulation of breast CSCs (Months 1-8)

Subtask 1: Establish BT549 cells that stably express miR-200c (pCDH-miR200c) and MCF7 cells with knock-down of miR-200c (pZIP-miR200c) (Months 1-2) **Completed**

We have successfully established stable miR-200c expression TNBC cell lines (MDA-MB-231-pCDH-miR200c, BT549-pCDH-miR200c), miR-200c knockdown luminal breast cancer cell lines (T47D-pZIP-miR200c, MCF7-pZIP-miR200c) and examined the protein expression levels as described in subtask 2.

Subtask 2: Determine expression levels of PKCζ and phospho-NUMB (p-NUMB), by re-expressing PKCζ in BT549-pCDH-miR200c cells and knocking-down PKCζ in MCF7-pZIP-miR200c cells (Months 3-5) **Completed**

We have successfully demonstrated that PKCζ is a bona fide miR-200c target. PKCζ protein expression and the phosphorylation level of its substrate p-NUMB are markedly upregulated by knock-down of miR-200c in T47D-pZIP-miR200c and MCF7-pZIP-miR200c cells, which can be reversed by knock-down of PKCζ. Consistently, PKCζ protein expression and the phosphorylation level of its substrate p-NUMB are downregulated by ectopic expression of miR-200c in MDA-MB-231-pCDH-miR200c and BT549-pCDH-miR200c cells, which can be rescued by re-expression of PKCζ (Fig. 1, data shown from T47D and MDA-MB-231 cells published in our Oncogene 36(22), 3193-3206).

Subtask 3: Determine changes in the percentage of CD24-CD44+ cells and the sphere forming capacity by ectopically expressing PKCζ in BT549-pCDH-miR200c cells and knocking-down PKCζ in MCF7-pZIP-miR200c cells (Months 6-8) **Completed**

We have successfully demonstrated that the percentage of CD24$^-$CD44$^+$ cells and the sphere forming capacity are markedly enhanced by knock-down of miR-200c, which can be reversed by knock-down of PKCζ. Consistently, the percentage of CD24$^-$CD44$^+$ cells and the sphere forming capacity are suppressed by ectopic expression of miR-200c, which can be rescued by re-expression of PKCζ (Fig. 2, published in our Oncogene 36(22), 3193-3206).

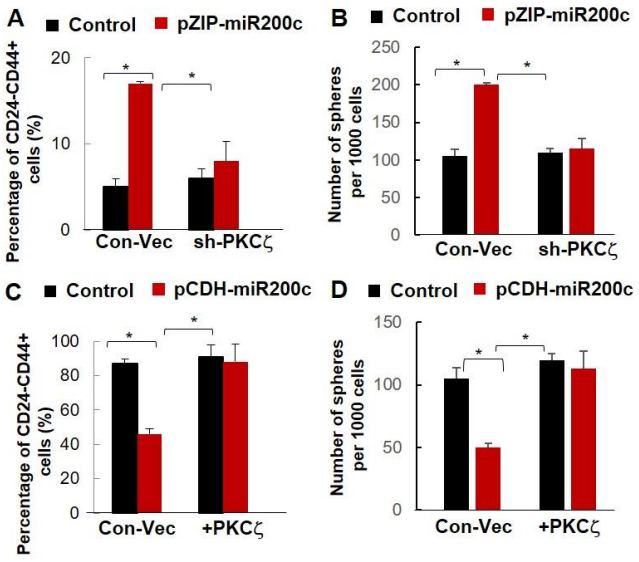
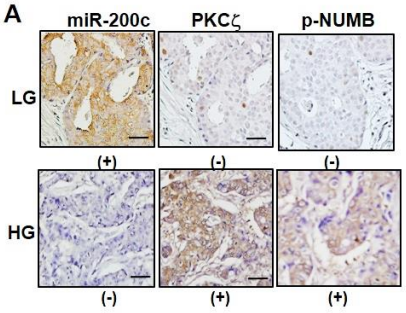


Figure 2. MiR-200c suppresses breast CSC traits through down-regulation of PKCζ. (A) The percentage of isolated CD24$^-$CD44$^+$ population, and **(B)** the number of tumor spheres (sphere size>100μm) per 1000 initially plated cells from MCF7-pZIP-miR200c cells that further expressed sh-PKCζ. **(C)** The percentage of isolated CD24$^-$CD44$^+$ population and **(D)** the number of tumor spheres (sphere size>100μm) per 1000 initially plated cells from BT549-pCDH-miR200c cells that further expressed PKCζ. n=3 independent experiments, asterisk indicates P<0.05. Error bars denote ±SD.

<u>Major Task 2: Determine the correlation of miR200c-PKCζ regulation with clinicopathological characteristics in human breast tissue samples (Months 9-14)</u>

Subtask 1: Determine the expression levels of miR-200c, PKCζ, p-NUMB by in situ hybridization or immunohistochemical staining in 150 human breast tumor samples (Months 9-12) **<u>Completed</u>**

We have performed a correlation analysis of PKCζ, p-NUMB, and miR-200c expression levels in human breast tissue specimens (Pantomics) consisting of a cohort of breast tumor samples. We have successfully demonstrated that PKCζ and p-NUMB levels are repressed in the well-differentiated low tumor grade breast tumors (LG, grade I), where miR-200c was highly expressed (Fig. 3, n=98). In contrast, the poorly-differentiated



B

Tumor Grade	LG	HG	
miR200c+	15 (15%)	10 (10%)	
miR200c-	11 (12%)	62 (63%)	
Total	26 (27%)	72 (73%)	*P=0.00001*
PKCζ+	7 (7%)	41 (42%)	
PKCζ-	19 (19%)	31 (32%)	
Total	26 (26%)	72 (74%)	*P=0.008*
pNUMB+	10 (10%)	42 (42%)	
pNUMB-	16 (17%)	30 (31%)	
Total	26 (27%)	72 (73%)	*P=0.01*

Figure 3. Lost miR200c expression is correlated with overexpression of PKCζ and p-NUMB in high-grade, aggressive breast cancer. (A) Representative IHC staining images showing expression levels of miR-200c, PKCζ and p-NUMB in 98 human breast tissue specimens, including low-grade tumors (tumor grade I, LG) and high-grade tumors (tumor grade II-III, HG, scale bar: 100μm). **(B)** Correlation of miR-200c, PKCζ and p-NUMB expression levels with tumor grade was analyzed by Chi-Square analysis. (-): negative-low staining, (+): strong-positive staining.

high tumor grade tumors (HG, grade II-III) exhibit overexpression of PKCζ and p-NUMB, along with significantly reduced miR-200c levels (Fig. 3, published in our Oncogene 36(22), 3193-3206).

Subtask 2: Correlation analysis of the protein levels among miR-200c, PKCζ, p-NUMB and their correlation with tumor subtype (p53, BRCA1, ER/PR/HER2 status), tumor grade (differentiation status), tumor stage (metastasis status), and 5 year recurrence status (Months 13-14) **Partially Completed**

We have shown enhanced PKCζ and p-NUMB expression levels are positively correlated with loss of ER in tumors (n=34, P=0.03), where miR-200c is also repressed (P<0.01). The expression pattern does not correlate with 5-year recurrence or metastasis status.

Specific Aim 2: Develop a therapeutic strategy targeting the cell polarity machinery to direct breast CSC fate

Major Task 3: Determine the role of miR200c-PKCζ signaling in regulation of the polarity of breast CSC division (Months 15-20)

Subtask 1: Confocal fluorescence paired cell imaging of CD44 and NUMB will be analyzed during the first division of the breast CSCs expressing the control vector, miR-200c, and miR-200c+PKCζ expression plasmids or under the treatment of vehicle/PKCζ inhibitor (Months 15-17)

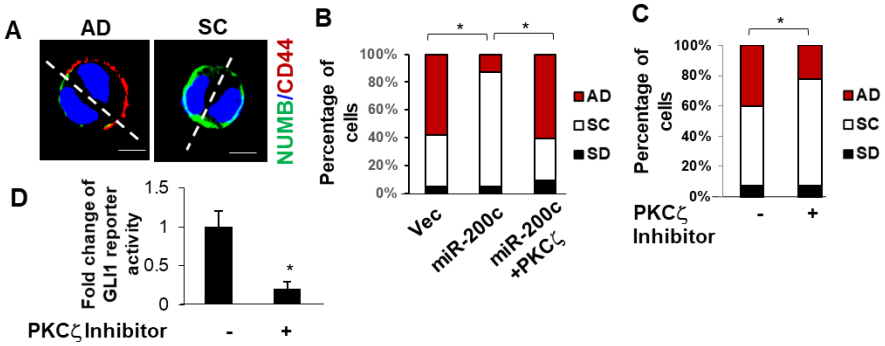


Figure 4. Inhibition of PKCζ leads to symmetric commitment of breast CSCs to favor the differentiation cell fate. (A) Representative images showing intracellular distribution of CD44 (red), NUMB (green) in the dividing stem cells during asymmetric cell division (AD) or symmetric lineage commitment (SC) of the $CD24^{-}CD44^{high}$-CSC population (scale bar: 10μm). **(B)** The percentage the CSCs that manifested specific cell division patterns in cells expressing miR-200c, miR-200c+PKCζ, or control vector. **(C)** Cell division patterns and **(D)** GLI1 reporter activity in cancer cells treated with PKCζ inhibitor or vehicle. n=3 independent experiments, asterisk indicates P<0.05. Error bars denote ±SD.

Completed

We have established and analyzed the paired cell image patterns (AD vs. SC) using CD44 and NUMB in the breast CSCs expressing miR-200c, and miR-200c+PKCζ expression plasmids or under the treatment of vehicle/PKCζ inhibitor. We have successfully demonstrated that inhibition of PKCζ leads to a markedly increased symmetric commitment (SC) along with a reduced asymmetric division (AD) shown in a representative AD vs. SC image (not specific to the treatment, Fig. 4A). We found that GLI1 signal shown in previous report using confocal imaging was not GLI-specific; here we revised measurement using GLI1 driven luciferse activity and showed decreased fold change of GLI1 reporter activity under PKCζ inhibitor treatment (Fig. 4D). Similar effects can be recapitulated by enforced expression of miR-200c, which are then reversed upon re-expression of PKCζ (Fig. 4).

Subtask 2: Serial sphere formation assay with repeated dissociation of spheres into single cells

followed by re-formation of spheres for three consecutive passages using breast CSCs expressing the control vector, miR-200c, and miR-200c+PKCζ expression plasmids or under the treatment of vehicle/PKCζ inhibitor (Months 18-20) **Completed**

We have established the sphere cultures expressing control vector, miR-200c, and miR-200c+PKCζ plasmids. The number of spheres per 1000 plated cells has been analyzed from three independent experiments of three serial passages. We have successfully demonstrated that inhibition of PKCζ leads to continuing diminishment of the spheres at each passage; similar effects can be recapitulated by enforced expression of miR-200c, which are then reversed upon re-expression of PKCζ (Fig. 5, 2017 Oncogene 36(22), 3193-3206).

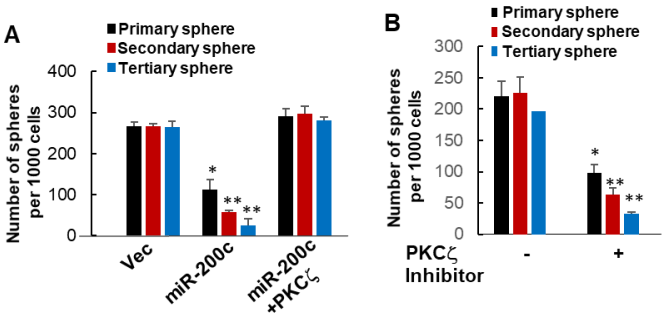


Figure 5. Inhibition of PKCζ leads to exhaustion of breast CSCs. (A) The number of the serially passaged tumor spheres from CSCs expressing miR-200c, miR-200c+PKCζ, or control vector, and from **(B)** the primary breast CSCs treated with PKCζ inhibitor or vehicle. n=3 independent experiments, asterisk indicates P<0.05; double asterisk indicates P<0.01 (compared with vector control group). Error bars denote ±SD.

Major Task 4: Determine the therapeutic effect of targeting miR200c-PKCζ signaling in vivo

Subtask 1: Determine the expected tumorigenic CSC frequency of primary breast CSCs stably expressing miR-200c, miR-200c+ PKCζ, and the control plasmids in mammary tumor xenograft animals using Extreme Limiting Dilution Analysis (Months 21-24) **Completed**

We have successfully demonstrated that down-regulation of PKCζ by miR-200c significantly inhibits mammary xenograft tumor formation with a significant reduction in the tumor-seeding CSC frequency, and these effects are reversed upon re-expression of PKCζ (Fig. 6A).

Subtask 2: Determine the therapeutic effect of PKCζ inhibitor on overcoming doxorubicin-resistance in NOD/SCID mice inoculated with primary breast CSCs stably expressing the GLI1-activity reporter (Months 25-28) **Ongoing**

We have successfully demonstrated the therapeutic efficacy of PKC inhibitor in suppression of breast tumor xenograft tumor (PDX) growth (Fig. 6B). We are still working on determining the maximum

A

miR-200c	-	+	+
PKCζ	-	-	+
$1x10^5$	5/5	3/5	5/5
$1x10^4$	4/5	2/5	4/5
$1x10^3$	4/5	1/5	3/5
$1x10^2$	3/5	0/5	2/5
CSC frequency	1/594	1/12,443*	1/723

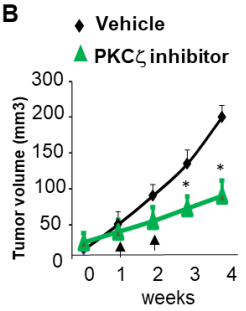


Figure 6. Inhibition of PKCζ effectively suppresses tumorigenic CSCs and xenograft tumor growth. (A) The calculated CSC frequency by the xenograft tumor formation in NOD/SCID mice inoculated with the indicated number of breast tumor cells that stably expressed miR-200c, miR-200c+PKCζ, or control vector using Extreme Limiting Dilution Analysis. **(B)** Tumor xenograft growth from NOD/SCID mice under PKCζ inhibitor (ip, 20 mg/kg) or vehicle treatment for two weeks (n=5, asterisk indicates P<0.05) Error bars denote ±SD.

tolerated dose and toxicity of the combination treatment (PKCζ inhibitor-doxorubicin) in tumor bearing NOD/SCID mice.

Major Task 5: Identify new lead compounds that impact the polarity of breast CSC division using phenotypic screening

Subtask 1: High throughput phenotypic screening (HTS) of compound libraries to identify the ones that direct primary breast CSCs to symmetric commitment (SC, SYC) by high content paired cell imaging analysis (Months 29-31) **Completed**

We have collaborated with Drug Discovery Core Facility to perform the paired cell analysis based-HTS screening. Hit compounds determined as > 2-fold increase in SYC division pattern or Z score > 5 were selected. All-trans retinoic acid (ATRA) was identified as one of the hits, and its role in directing CSC to SYC fate has been validated in our recent publication (Oncogene 36(22), 3193-3206).

Subtask 2: Determine the dose response curve by exposing the primary breast CSCs to the candidate SC-promoting compounds identified from subtask1 at twelve different concentrations with two-fold serial dilutions between 0 and 100μM in triplicate using non-linear regression analysis (Prism software) (Months 32-33) & Subtask 3: Serial sphere formation assay with repeated dissociation of spheres into single cells followed by re-formation of spheres for three consecutive passages using the primary breast CSCs under the treatment of the hit compounds identified from subtask1 with the effective concentrations (EC50) confirmed in subtask 2. (Months 34-36) **Ongoing**

We are currently analyzing the very large preliminary datasets generated from the proposed high throughput compound screening experiment that have taken longer than expected to be analyzed. The hits will then be validated as proposed in subtask 2 and 3.

- **Opportunities for training and professional development:**

  1. The award provides training opportunities to the postdoc fellow, Yu-Syuan Chen, and the graduate student, Meng-Ju Wu, to receive courses, mentoring, and research experience that have advanced their professional skills. Meng-Ju Wu has completed his PhD and a new graduate student will continue the study.

  2. The award provides professional development opportunities to the graduate students, Meng-Ju Wu and Yingsheng Zhang, for participation in conferences, such as 2018 American Association of Cancer Research Annual Conference.

- **Results disseminated to communities of interest:**
  Nothing to report.

- **Plan to do during the next reporting period to accomplish the goals:**
  We have successfully completed the proposed studies in Aim1 and Aim2 with the results that are highly supportive of our central hypothesis. We will continue analyzing the large datasets generated from the proposed high throughput compound screening experiment and validating the SC-promoting compounds in regulation of CSC capacity as planned in the major task 5 during the next reporting period. A no-cost extension has been approved to change the project expiration date to September 29, 2019 that would allow us to conclude all the proposed work, and have the final data incorporated into a manuscript for publication.

4. Impact

The impact on the development of the principal discipline(s) of the project:

About 1 in 8 U.S. women will develop breast cancer over the course of her lifetime, and in the year of 2018, breast cancer has claimed the lives of more than 40,000 women and men in the United States. Although initial remission can be achieved with chemo-treatments, the worry and fear of treatment resistance, recurrence, and death still have a deep impact on many breast cancer patients. It is recognized that cancer stem cells (CSCs), a long-lived, self-perpetuating cell population that can infinitely give rise to the bulk of a tumor as the "seed" of the cancer, account for cancer initiation, progression, radio-/chemo-resistance, and recurrence. To date, treatment strategies designed to eliminate the genesis of the cancer (CSC) still remain a significant challenge. This project aims to identify critical cell components and their working mechanisms that are used to sustain the breast CSC pool, and the identified mechanism will further be therapeutically targeted to direct CSCs to a terminally dormant cell fate and become sensitive towards radio-/chemo-therapy. With the common properties of CSCs between many cancer types, we believe that the applications generated from our research will continuingly contribute to overcoming the therapeutic hurdles of a broad spectrum of cancers and significantly benefit the cancer patient and the survivor community for decades.

The impact on other disciplines:

Nothing to report.

The impact on technology transfer:

Nothing to report.

The impact on society beyond science and technology:

Nothing to report.

5. Changes/Problems

The project was not able to be completed during original grant period because the very large datasets generated from the proposed high throughput compound screening experiment in the major task 5 have taken longer than expected to be analyzed and validated, and a student who works on the project has graduated and left the lab. A no-cost extension has been approved to change the project expiration date to September 29, 2019. This extension period is required to allow us to conclude all the proposed work, and have the final data incorporated into a manuscript for publication. There will be no change in the project's originally approved scope of work.

6. Products

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

(1) Journal publications (#: corresponding author):

a. Wu MJ, Kim M, Chen YS, Yang JY, and Chang C-J[#] (2017) Retinoic acid directs breast cancer cell state changes through regulation of TET2-PKCζ pathway. **Oncogene Jun1; 36(22), 3193-3206** (**acknowledgement of federal support- Yes**)

(2) Presentations:

a. 2018 American Association of Cancer Research Annual Conference. "The role of Tet2 in mammary stem cell fate decision"

- **Technologies, inventions, patent applications, and/or licenses:**
Nothing to report.

- **Other Products:**
a. Establishment and validation of MDA-MB-231-pCDH-miR200c and BT549-pCDH-miR200c cells that stably express miR-200c and T47D-pZIP-miR200c and MCF7-pZIP-miR200c cells that stably knock-down for miR-200c.

b. Generation of ChIP-sequencing and microarray datasets (GSE85189, GSE85141) as reported in the Oncogene paper (6-1-a).

7. Participants & Other Collaborating Organizations

- **Individuals and other support:**
  Nothing to report.

- **Other involved organizations:**
  Nothing to report.

8. Special Reporting Requirements

Nothing to report.

9. Appendices

Nothing to report.