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PRINCIPAL INVESTIGATOR: Qiang Zhou

CONTRACTING ORGANIZATION: University of California, Berkeley
Berkeley, CA 94704

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Qiang Zhou

E-Mail: qzhou@berkeley.edu

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14. ABSTRACT:
This project focuses on the important but under-studied role of the P-TEFb-dependent transcription elongation machinery in human breast cancer progression. It aims to test the hypothesis that transcription elongation is a key regulatory step in breast cancer development, and that targeting P-TEFb can be an effective strategy to block breast cancer progression. During the current reporting period, we have made significant progress toward the identification of the ELL2-containing SEC as the form of active P-TEFb that plays a key role in promoting breast cancer cell EMT. Through disrupting the negative P-TEFb complex, the 7SK snRNP, by targeted knockdown of HEXIM1 expression, we found that the KD not only promotes EMT but also enhances the interaction of CDK9 with HSP90, which has been strongly implicated in tumorigenesis. With this new information, we are investigating the mechanisms and significance of the ELL2-SEC and the CDK9-HSP90 complex in controlling the expression of key EMT and stemness regulators to accomplish the stated goals of the project in the next reporting period.

15. SUBJECT TERMS

Nothing listed

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1. INTRODUCTION:

Aberrant gene expression, caused by mutations in various signaling pathways, lie in the heart of breast cancer development and progression. Mammalian gene expression is controlled primarily at the level of transcription, which consists of several closely interlinked stages. During the past 30 years, the transcription field has been pre-occupied with the pre-initiation and initiation stages of transcription and ignored the subsequent elongation step, which in recent years has been shown to be extremely critical for the control of cell growth, embryonic development, as well as stem cell self-renewal and differentiation. This proposal focuses on the important but under-studied role of the transcription elongation machinery in human breast cancer progression and is designed to test the hypotheses that a network of P-TEFb-containing elongation complexes plays a key role in regulating breast cancer EMT, stemness, invasion and metastasis through controlling the expression of essential EMT and metastasis regulators, and that targeting P-TEFb is a viable therapeutic approach to halt breast cancer progression.

At the core of the elongation machinery is the CDK9 and cyclin T1 heterodimer termed P-TEFb that stimulates the transition of RNA Pol II from promoter-proximal pausing to productive elongation by phosphorylating Pol II and antagonizing negative elongation factors. In mammalian cells, P-TEFb is maintained in a functional equilibrium between the active and inactive states through reversible associations with distinct regulators that collectively form a network of P-TEFb complexes. Under normal growth conditions, more than half of nuclear P-TEFb are sequestered in a kinase-inactive complex called the 7SK snRNP that contains the 7SK snRNA as a structural scaffold, HEXIM1 as the kinase inhibitor, and LARP7 and MePCE as proteins that bind to and maintain the stability of 7SK snRNA. The 7SK snRNP represents the principle cellular reservoir of uncommitted P-TEFb and responds to demands for increased transcription and cell proliferation by releasing P-TEFb, which can subsequently be recruited by Brd4 to chromatin templates or integrated into the Super Elongation Complex (SEC) for transcriptional activation. The bromodomain protein Brd4 recruits P-TEFb to chromatin templates through interacting with acetylated histones and the mediator complex and is required for transcription of many primary response and signal-induced genes. In addition to P-TEFb, the SEC contains mostly fusion partners (e.g. AFF1, AFF4, ELL1, ELL2, ENL and AF9) of the mixed lineage leukemia (MLL) protein and promotes transcription of MLL-target genes, leading to some of the most severe forms of leukemia. Our working hypotheses is that P-TEFb activation as a result of shifting its functional equilibrium to the active side is a major driving force to promote breast cancer EMT, stemness and metastasis, and that the interference of this activation halts cancer progression and can thus be an effective therapy.

2. KEYWORDS:

Transcriptional elongation, P-TEFb, breast cancer, epithelial to mesenchymal transdifferentiation (EMT), invasion, metastasis

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Two specific aims have been proposed: 1) Determine whether the P-TEFb functional equilibrium can be perturbed to affect breast cancer EMT, invasion and metastasis, and whether a small molecule inhibitor of P-TEFb can be employed to halt breast cancer progression; 2) Determine why the EMT and metastasis-related genes are particularly sensitive to transcription elongation control and P-TEFb availability.

Research-Specific Tasks:

Major Task 1: Specific Aim 1: Determine whether the P-TEFb network can be manipulated to suppress breast cancer EMT, invasion and metastasis.	**Months**	**Researcher**	**Percentage completion**
Subtask 1: Determine the roles of P-TEFb complexes in breast cancer EMT, stemness and metastasis in vivo. Overexpression or shRNA-based knockdown of various components of the P-TEFb complexes will be performed in breast cancer cells, and the effects of these manipulations on breast cancer EMT and metastasis will be determined in vitro and in vivo.	1-30	H. Shao (Luo) H. Lu (Zhou)	80%
Subtask 2: Determine whether small molecule CDK9 inhibitors can be used to halt breast cancer metastasis. 8 experimental groups to test various drug dosage and frequency regimes will be tested in vivo. An additional 8 experimental groups for tumor maintenance experiment and 3 groups for orthotopic experiment will be included.	7-36	H. Shao (Luo)	20%
Major Task 2: Specific Aim 2: Determine whether and why the EMT and metastasis-related genes are particularly sensitive to transcription elongation control and P-TEFb availability.	**Months**	**Researcher**	**Percentage completion**
Subtask 1: Determine which SEC complex(es) mediates activation of EMT genes in breast cancer cells.	6-30	H. Lu (Zhou)	80%
Subtask 2: Determine the molecular basis underlying high sensitivity of EMT and metastasis-related genes to control at the transcription elongation stage.	12-36	H. Lu (Zhou)	20%

What was accomplished under these goals?

Specific Aim 1: Determine whether the P-TEFb network can be manipulated to suppress breast cancer EMT, invasion and metastasis.

Subtask 1: Determine the roles of P-TEFb complexes in breast cancer EMT, stemness and metastasis in vivo.

We have previously shown that the depletion of LARP7, a key component of the P-TEFb-containing 7SK snRNP, in non-invasive breast cancer cells promotes epithelial to mesenchymal transdifferentiation (EMT) and metastasis by upregulating key EMT/metastasis-related genes (Ji et al. eLife, 2014). These data have for the first time implicated a direct role for the P-TEFb transcription elongation machinery in breast cancer progression. However, in addition to residing in the 7SK snRNP, LARP7 can also exist in other nuclear complexes and can potentially participate in other unrelated biological functions. It is thus essential to further confirm that it is indeed the P-TEFb-containing 7SK snRNP that is intimately involved in the control of breast cancer progression.

To accomplish this goal, one important task we have undertaken during the past year is to disrupt 7SK snRNP by targeting HEXIM1, which is another key component of the 7SK snRNP as well as an inhibitor of the P-TEFb kinase within the complex, in non-invasive breast cancer T47D cells and then to evaluate the impact on breast cancer EMT and metastasis. In the wound-healing assay, the HEXIM1 knockdown (KD, Fig. 1A) cells exhibited markedly faster wound closure than the control cells (Fig. 1B). Consistently, the KD also accelerated cell migration in Transwell assays (Fig. 1C). Together, these data have confirmed from the HEXIM1 angle that altering the P-TEFb network by disrupting the inactive 7SK snRNP can indeed promote breast cancer EMT and progression.

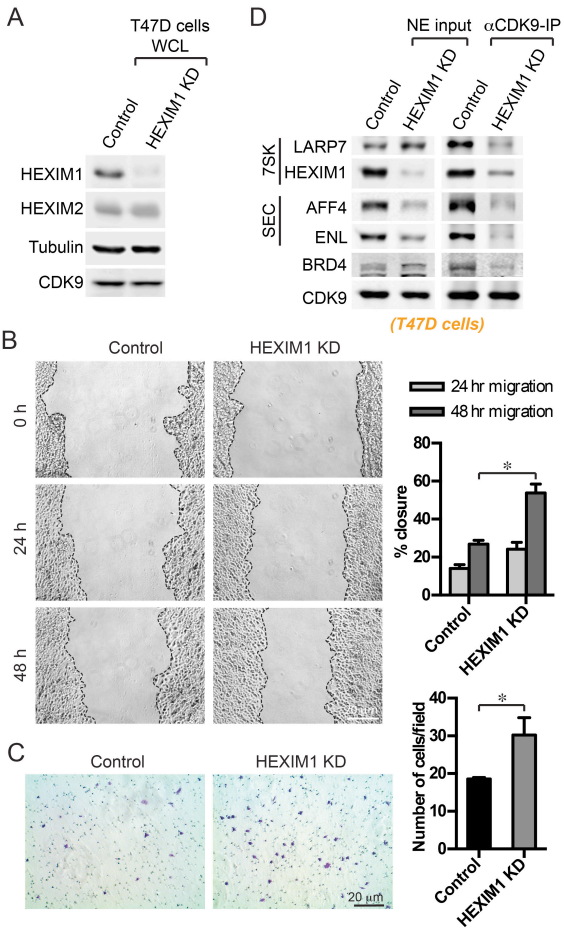

Fig. 1. Knockdown (KD) of HEXIM1 in T47D cells enhances breast cancer EMT and alters P-TEFb network. (A). Western blotting shows HEXIM1 KD efficiency in T47D cells that stably express shHEXIM1. **(B).** Wound healing assay. Confluent cell monolayers were wounded, and wound closure was monitored at 24 hr and 48 hr. **(C).** Migration assay. Control and HEXIM1 KD T47D cells were subjected to a Transwell migration assay. (D). Nuclear extracts were prepared from control and HEXIM1 KD cells and subjected to anti-CDK9 IP. The CDK9-bound proteins were analyzed by western blotting. Data are presented as mean ± SE from three independent experiments.

Very surprisingly, we have found that the HEXIM1 KD not only released P-TEFb from 7SK snRNP, but also reduced the interactions of P-TEFb with the SEC components as well as BRD4 (Fig. 2D). This is clearly different from the situation involving the KD of LARP7, where the disruption of 7SK snRNP by the KD caused the transfer of P-TEFb to the SEC and BRD4 (Ji et al. eLife, 2014). To find out what P-TEFb is up to in the HEXIM1 KD cells, we performed affinity-purification and mass spectrometric analysis and our preliminary data indicate that the P-TEFb component CDK9 had significantly enhanced interaction with the molecular chaperone heat shock protein HSP90 upon the KD.

Recently, HSP90 has gained a lot of attention in the cancer field due to its ability to facilitate the function of numerous oncoproteins in tumor cells, which are said to be 'addicted' to this protein. Consistently, pharmacological inhibition of Hsp90 has demonstrated great promise in cancer treatment. We are currently investigating the biological significance as well as the mechanism by which the HEXIM1 depletion promotes the CDK9-HSP90 complex formation. We are also testing whether the combinatorial use of both the CDK9 and HSP90 inhibitors can lead to synergistic inhibition of the transcription of EMT and metastasis-related genes, which can potently suppress breast cancer progression and metastasis.

Specific Aim 2: Determine whether and why the EMT and metastasis-related genes are particularly sensitive to transcription elongation control and P-TEFb availability.

Subtask 1: Determine which SEC complex(es) mediates activation of EMT genes in breast cancer cells.

1. The SEC component ELL2 is up-regulated in invasive breast cancer cell lines

We have previously shown that the depletion of the 7SK snRNP component LARP7 causes the disruption of the snRNP and release of P-TEFb, which is then converted into two active P-TEFb complexes, the SEC and the BRD4-P-TEFb complex, resulting in the promotion of breast cancer progression (Ji et al. eLife, 2014). However, due to the fact that all subunits within a SEC have their functional homologues in human cells, the different combinations among these homologues can generate a fairly large family of related complexes with largely non-overlapping functions. Another major progress we have made during the current reporting period is the determination of whether any particular members of the SEC family may exert a predominant role in promoting breast cancer progression and metastasis.

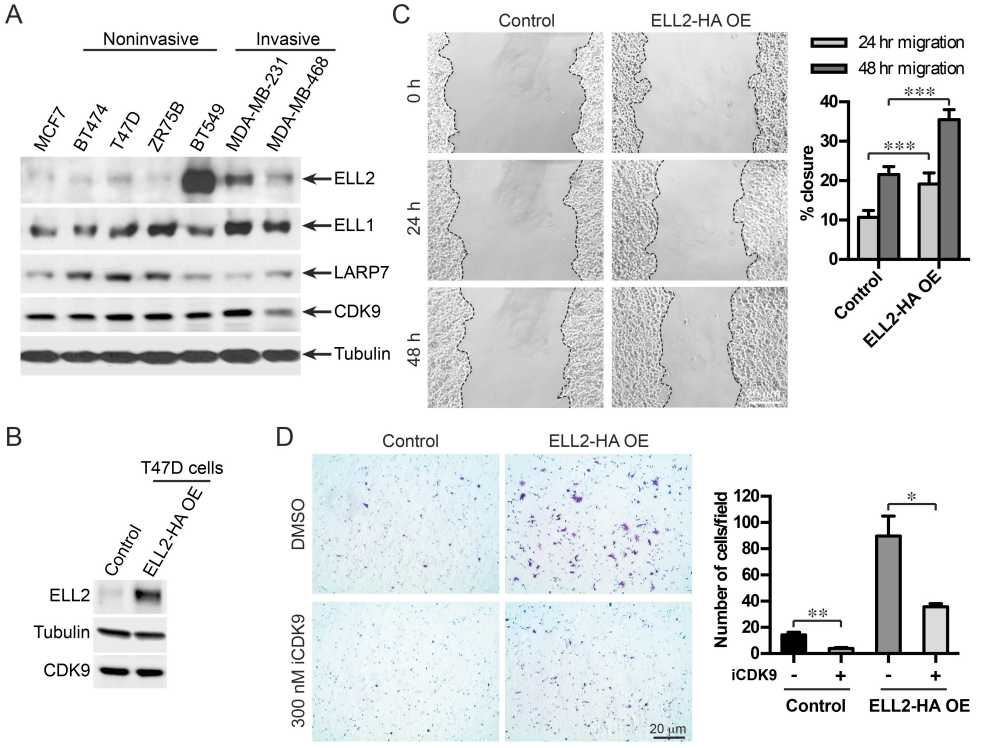

Fig. 2. Upregulation of ELL2 promotes EMT of T47D breast cancer cells. (A). Levels of ELL2, ELL1, LARP7 and CDK9 in non-invasive and invasive breast cancer cell lines are analyzed by western blotting. **(B).** Overexpression level of ELL2 in T47D cells. **(C).** Wound-healing assay. Confluent cell monolayers were wounded, and wound closure was monitored at 24 hr and 48 hr. **(D).** Migration assay. Control and ELL2 overexpressing T47D cells were treated with i-CDK9 for 20 hr and subjected to a Transwell migration assay. Data are presented as mean ± SE from three independent experiments. OE indicates overexpression.

Toward this goal, we first examined the expression levels of ELL1 and ELL2, two homologous SEC subunits that reside in separate SEC complexes but can directly stimulate RNA polymerase (Pol) II elongation

by suppressing transient pausing of Pol II, among a panel of well-characterized breast cancer cell lines. We found that ELL2 but not ELL1 was expressed at a notably higher level in invasive breast cancer cell lines than in non-invasive cell lines (Fig. 2A), suggesting that the ELL2-containing SEC may play a key role in promoting breast cancer progression.

2. **ELL2 promotes breast cancer EMT in a P-TEFb-dependent manner**

To further investigate the role of the ELL2-containing SEC in breast cancer development, we overexpressed ELL2 in non-invasive T47D cells (Fig. 2B). Our data show that the ELL2 up-regulation in T47D cells dramatically enhanced cell motility and migration (Fig. 2C and 2D), indicating an increased EMT. Notably, these effects induced by ELL2 overexpression were partially reversed by the treatment of cells with the P-TEFb inhibitor i-CDK9 (Fig. 2D), suggesting that the observed increase in EMT in ELL2-overexpressing cells was due to the elevated P-TEFb activity.

Subtask 2: Determine the molecular basis underlying high sensitivity of EMT and metastasis-related genes to control at the transcription elongation stage.

Most of the reagents including cell lines that are necessary for this subtask have been established and the experimental conditions have been tested and verified. The actual experiments will be starting in year 3.

In summary, after establishing key reagents and experimental conditions in the prior year, we have now performed during the current reporting period several key experiments that are designed to test and confirm the hypothesis that the P-TEFb functional network, especially the 7SK snRNP, can be targeted to promote breast cancer EMT and metastasis. Furthermore, we have began to identify the precise form of the active P-TEFb-containing SEC complex that is especially important for the stimulation of the key EMT genes. These experiments have also revealed unexpected new implications for the possible involvement of HSP90 in breast cancer progression together with P-TEFb. These observations will provide a solid foundation for us to continue to make excellent progress towards achieving the remaining goals of the project during the next reporting period.

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

This project represents a major focus of research conducted in both the Zhou and Luo laboratories. It applies the concepts and experimental techniques derived from multiple disciplines and thus offer Dr. Hengyi Shao and Dr. Huasong Lu, two new postdoctoral researchers recently joined our laboratories, an excellent training opportunity to become exposed to and familiar with the languages and tools used in the areas of biochemistry, molecular cell biology, and bioinformatics. Through supervising, training, coordinating, recruiting, motivating, writing and defining research directions for all specific aims, Dr. Zhou and Dr. Luo, the principal investigators of these two partnering awards have been intimately involved in every aspect of the project. In addition, the two PIs have taught beginning graduate students to set up experiments, and used the weekly joint lab meetings and journal clubs as opportunities to train the students and postdoctoral researchers to better organize their data and thoughts and give more succinct and impressive presentations.

The scientific environment at UC Berkeley, where this project is being performed, also provides excellent opportunities for intellectual growth and collaboration for the researchers associated with this project. Many regularly scheduled seminars encompassing all areas of modern biology are available and can benefit this project. The MCB Department and the Division of Biochemistry and Structural Biology and Division of Cell and Developmental Biology, to which the Zhou and Luo laboratory belongs respectively, organize annual retreats where graduate students and postdoctoral researchers from the two laboratories have opportunities to present their latest findings and obtain valuable feedbacks. Additional interactions are frequent between our two

labs and those of Drs James Hurley, Britt Glaunsinger, Robert Tjian, Jennifer Doudna, Michael Botchan, and Michael Rapé, among others, and provide further intellectual support, technical help with experiments and useful reagents/tools. Moreover, many UC Berkeley labs are at the forefront of technology innovation, providing the researchers in the two labs with an opportunity to take advantage of the best new methods in proteomics, computational, imaging, genomic editing, and structural analyses. In summary, the breadth and depth of the UC Berkeley scientific environment where the Zhou and Luo laboratories are located provide unparalleled opportunities for training and professional development for all the researchers working on this project.

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?

During the next reporting period, we will continue to investigate the mechanism by which P-TEFb activation promotes breast cancer invasion and metastasis. In light of the new information obtained during this reporting period, we plan to expand the goals in Aim 1 to include the determination of the precise mechanism and physiological significance of the HEXIM1 KD-induced CDK9-HSP90 interaction in breast cancer cells. We will also test the hypothesis that simultaneous targeting both CDK9 and HSP90 will produce a synergistic effect to suppress the expression of key EMT genes to inhibit breast cancer invasion and metastasis. For Aim 2, a major focus will be placed on the determination of the molecular mechanism by which the ELL2-containing SEC activates transcriptional elongation of master EMT regulators. In addition, the requirement for both the ELL2-SEC and the BRD4-P-TEFb complex in breast cancer progression will be investigated by using the engineered cell lines generated during the previous reporting period as well as several BRD4 inhibitors we have obtained. Finally, we plan to identify the direct P-TEFb/SEC-target genes among those encoding the various EMT transcription factors and CSC stemness factors. Once this information is obtained, genome-wide ChIP-seq assay will be performed to evaluate the pausing index for RNA Pol II. The potential role of a Super Enhancer will also be tested by knocking down the mediator complex in these breast cancer cells and test whether this blocks breast cancer progression. Taken together, with the establishment of key reagents and experimental conditions and several pieces of new information obtained so far, we are on track to test the hypotheses put forward in the original proposal and also expand our studies to break new ground.

4. IMPACT:

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

The successful completion of the project will not only establish a new conceptual paradigm, but also have important clinical implications in halting breast cancer progression and metastasis. Specifically, our study will confirm the components of the general transcription elongation machinery as an important factor to drive the metastasis of breast cancer. By employing a novel highly selective P-TEFb inhibitor in the proposed experiments, we will also directly test the idea that targeting the P-TEFb network of complexes can be an effective strategy to treat metastatic breast cancer.

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.

5. CHANGES/PROBLEMS:

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.

6. PRODUCTS:

Publications, conference papers, and presentations

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

New breast cancer stable cell lines have been established. They will be freely shared with the scientific community upon request.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Qiang Zhou
Project Role:	Initiating PI
Researcher Identifier:	qzhou (eCommon ID)
Nearest person month worked:	4
Contribution to Project:	Dr. Zhou supervises, trains, recruits, and motivates all personnel on the project. He also coordinates and defines research directions for all specific aims together with Dr. Kunxin Luo, the partnering PI
Funding Support:	This award

Name:	Hengyi Shao
Project Role:	Postdoctoral researcher
Researcher Identifier:	Hengyishao (eCommon ID)
Nearest person month worked:	6
Contribution to Project:	Dr. Shao has performed various EMT, cancer stemness and invasion assays to test whether activation of P-TEFb leads to EMT and metastasis of breast cancer cells and discovered Hsp90 as the associated protein of P-TEFb.
Funding Support:	This award

Name:	Huasong Lu
Project Role:	Postdoctoral researcher
Researcher Identifier:	Luhuasong (eCommon ID)
Nearest person month worked:	1
Contribution to Project:	Dr. Lu has participated in the generation of all the cell lines stably knocking out the components of various P-TEFb complexes and performed some of the biochemical experiments assessing the changes in P-TEFb complex formation upon knocking down or overexpression of various components.
Funding Support:	NIH

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?

None.

8. SPECIAL REPORTING REQUIREMENTS:

An independent report will be submitted by the partnering PI Dr. Kunxin Luo.

9. APPENDICES:

None