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Award Number: W81XWH-10-1-0316

TITLE: Epigenetic Alterations Associated With CCCTC-Binding Factor Deregulation  
in Prostate Cancer

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CONTRACTING ORGANIZATION:

(Enter the Name, City, State and Zip Code of the Contracting Organization)

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REPORT DATE: September 2013

TYPE OF REPORT: Annual summary

PREPARED FOR: U.S. Army Medical Research and Materiel  
Command

Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

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| REPORT DOCUMENTATION PAGE                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |                  |                                  |                               | Form Approved<br>OMB No. 0704-0188              |                                              |
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| 1. REPORT DATE : Sept 2013                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |                  | 2. REPORT TYPE<br>ANNUAL Summary |                               | 3. DATES COVERED<br>1 July 2010 to 30 June 2013 |                                              |
| 4. TITLE AND SUBTITLE<br><br>Epigenetic Alterations Associated With CCCTC-Binding Factor<br>Deregulation in Prostate Cancer                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |                  |                                  |                               | 5a. CONTRACT NUMBER                             |                                              |
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| 6. AUTHOR(S)<br><br>Jin-Hee Lee, Ph.D.<br><br>Email: jhlee38@wisc.edu                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 |                  |                                  |                               | 5d. PROJECT NUMBER                              |                                              |
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| 7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)<br>University of Wisconsin-<br>Madison<br>Madison, Wisconsin 53706-1490                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |                  |                                  |                               | 8. PERFORMING ORGANIZATION REPORT<br>.....      |                                              |
| 9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)<br><br>U.S. Army Medical Research and Materiel<br>Command<br>Fort Detrick, Maryland 21702-5012                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |                  |                                  |                               | 10. SPONSOR/MONITOR'S ACRONYM(S)                |                                              |
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| 12. DISTRIBUTION / AVAILABILITY STATEMENT<br>Approved for public release; distribution unlimited                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |                  |                                  |                               |                                                 |                                              |
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| 14. ABSTRACT<br>CCCTC-binding factor (CTCF) is a widely expressed 11-zinc finger nuclear protein. CTCF plays an important role in epigenetic regulation by establishing, maintaining epigenetic states in the genome, preventing the spread of DNA methylation, and maintaining methylation-free zones important in gene transcription. CTCF regulates multiple genes involved in cell cycle progression and growth deregulation in both normal and prostate cancer cells. Thus, it serves as a 'master regulator' that when functionally altered has the potential to globally change the behavior of a cell. It is our hypothesis that decreased CTCF expression in prostate cancer modulates the expression of growth promoting and tumor suppressor genes and may be an early permissive change in prostate cancer. Decreased CTCF expression would be associated with changes in local and global methylation especially at DNA binding sites of CTCF target genes in prostate cancer. <u>Specific Aims:</u> (1) Determine if decreased expression of CTCF leads to the deregulation of growth regulatory genes in human prostate epithelial cells and prostate cancer cells. (2) To test if decreased expression of CTCF leads to changes in local and global methylation. (3) Determine CTCF/BORIS expression or function in prostate cancer can predict clinical outcomes. To date, CTCF has not been examined in the context of prostate cancer and no studies have been done studying the effect of CTCF deregulation in normal or nontumorigenic cells. Given its role as a 'master regulator' of multiple genes, this project has the potential to discover a new potential therapeutic target for future studies. Furthermore, CTCF could be used as a marker for prognostic and diagnostic purposes in prostate cancer. |                  |                                  |                               |                                                 |                                              |
| 15. SUBJECT TERMS<br>CCCTC-binding factor (CCTF), DNA methylation, Epigenetics                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |                  |                                  |                               |                                                 |                                              |
| 16. SECURITY CLASSIFICATION OF:                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |                  |                                  | 17. LIMITATION<br>OF ABSTRACT | 18. NUMBER<br>OF PAGES                          | 19a. NAME OF RESPONSIBLE PERSON              |
| a. REPORT<br>U                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        | b. ABSTRACT<br>U | c. THIS PAGE<br>U                |                               |                                                 | USAMRMC                                      |
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## **INTRODUCTION:**

*CCCTC-binding factor (CTCF)* is a widely expressed 11-zinc finger nuclear protein originally identified as a transcription factor that binds to avian and mammalian MYC promoters (1). CTCF plays an important role in epigenetic regulation by establishing and maintaining epigenetic marks at multiple sites in genome (2). CTCF also prevents spreading of DNA methylation and plays a critical role in maintaining methylation-free zones (3). CTCF has been implicated in control of allele-specific gene expression on imprinted loci and of growing list of promoters involved in cell cycle control, differentiation and apoptosis. Chromosomal band 16q22.1, to which CTCF has been mapped, is frequently found to be deleted in sporadic breast and prostate tumors (4). The genetic loss of CTCF may be involved in dysregulation of number of oncogenes and tumor suppressor genes, including MYC, Rb, PIM1, BRCA1, and IGF2 suggesting that deregulation of CTCF can itself be implicated in cancer development (2). In an analysis of 344 samples of invasive breast carcinoma by differential immunocolocalization, revealed that CTCF abnormally locates in the cytoplasm of 77% of the cases (5). This suggests an important role for the gene in cancer progression and may represent a common feature in cancer cells. The prevailing thought is that reduction of CTCF levels predisposes the cell to epigenetic deregulation at multiple CTCF sites in the genome, inducing progressive silencing of tumor suppressor genes (2). Thus, CTCF could be a 'master regulator' controlling the epigenetic regulation of number of growth and tumor suppressor genes. Previous CTCF work in our laboratory has shown that CTCF declines with senescence using cultured prostate human epithelial cells *in vitro* (6), as well as in aging mouse prostate *in vivo* (7). It is our hypothesis that decreased CTCF expression in prostate cancer modulates the expression of growth promoting and tumor suppressor genes and may be an early permissive change in prostate cancer. Decreased CTCF expression would be associated with changes in local and global methylation especially at DNA binding sites of CTCF target genes in prostate cancer. Additionally, we hypothesize that decreased CTCF expression or function would be a negative prognostic marker for prostate cancer patients.

## **BODY:**

Note: In October 2011 Dr Bhusari left the institution to take a position as a scientist in academics at the NIEHS in North Carolina. In September 2012 Dr Jin-Hee Lee became the PI of the grant and will finish the proposal through an approved extension. Therefore, there has not been significant progress on the proposal in the last year. However, we have updated progress to date on work for the last month.

### **Task 1: Determine if decreased expression of CTCF leads to deregulation of growth regulatory genes in human prostate epithelial cells and prostate cancer cells. (Months 1-12)**

#### **a. Establish cell cultures of human prostate cancer (PC-3 and PPC-1) cell lines, HPECs, non-tumorigenic HPV16 E6 and/or E7 prostate cell lines.**

We have had to reestablish stable cell lines containing inducible multiple CTCF shRNA in pTRIPZ vector in PPC-1, LNCaPs, 293T and non-tumorigenic HPV16 E6

and/or E7 prostate cell lines. We have had to rederive these due to leakage from the promoter leading to clonal selection.

**b. Conduct CTCF shRNA knockdown experiments in above cell cultures.**

We are conducting inducible CTCF shRNA knockdown experiments in the above cell lines. CTCF shRNA was cloned in inducible pTRIPZ lentiviral vector system (Open Biosystems). Expression of CTCF shRNA is controlled by addition of Doxycycline (2µg/ml) in cell culture media for 3-5 days. Doxycycline treated cells were harvested after 3-5 days and CTCF knockdown was measured using western blots. We see efficient knockdown of CTCF expression in these cells after induction of CTCF shRNA with doxycycline (data not shown).

**c. Conduct quantitative RT-PCR analysis for PTEN, NKX3.1, WT1 and P57 (this has been accomplished for ARF, Rb genes) and western blot analysis.**

We are in the process of analyzing target gene expression changes in response to CTCF knockdown in above cell lines using quantitative RT-PCR and western blots.

**d. Perform proliferation, cell cycle assays using CTCF shRNA transfected cells.**

Proliferation in CTCF knockdown cells was measured as BrdU incorporation into the DNA of cells actively in S phase of the cell cycle.  $1.0 \times 10^5$  cells are seeded into 6-well plates and cultured for 3-5 days in growth medium (DMEM + 10% FBS) at 37°C in 5% CO<sub>2</sub> with or without doxycycline. After this initial culture period, 20 mM BrdU is added to cell-culture medium, 30 min prior to trypsinization and fixed in cold 95% ethanol. These cells are then rehydrated and stained for BrdU and cell cycle phases analyzed using flow cytometry. In LNCaP cells with CTCF knockdown there was significant decrease in percentage of cells in S phase indicating a decrease in cell proliferation in response to CTCF ablation in these cells. Percentage BRDU incorporation was also significantly reduced in P1 anti-CTCF shRNA expressing cells indicating that CTCF ablation results in decrease in LNCaP cell proliferation.

In PPC-1 cells with CTCF knockdown there was significant decrease in percentage of cells in S phase indicating a decrease in cell proliferation in response to CTCF knockdown in these cells. Percentage BRDU incorporation was also significantly reduced in P1 anti-CTCF shRNA expressing cells indicating that CTCF ablation results in decrease in PPC-1 cell proliferation.

We analyzed effects of CTCF knockdown in 293T cells which express high levels of endogenous CTCF expression. We made 293T stable cell line expressing anti-CTCF shRNA and scrambled control shRNA. In 293T cells with CTCF knockdown there was significant decrease in percentage of cells in S phase indicating a decrease in cell proliferation in response to CTCF knockdown in these cells. Percentage BRDU incorporation was also significantly reduced in P1 anti-CTCF shRNA expressing cells indicating that CTCF ablation results in decrease in PPC-1 cell proliferation.

**e. Generate CTCF shRNA lentivirus.**

We have generated shRNA lentivirus containing CTCF specific shRNA multiple constructs, empty pTRIPZ vector and control scrambled shRNA.

**f. To test the tumorigenic ability of CTCF shRNA infected non-tumorigenic E6/E7 cells using colony forming assays and tumor xenograft mouse models.**

Planned.

**Task 2. To determine if decreased CTCF expression leads to changes in local and global methylation. (Months 4-18).**

a. Infect HPECs, E6/E7 and prostate cancer cells with CTCF shRNA. We have done this experiment by generating CTCF shRNA containing stable cell lines in prostate cancer cells (LNCaP, PPC-1), non-tumorigenic E6/E7 cells. We are conducting transient transfection experiments in HPECs as reported in task1.

**b. Determine whether decreased CTCF leads to changes in global methylation using methyl-acceptor assay.**

We are in process of conducting these experiments (Months 12-18).

**c. Perform methylation mapping and quantitative methylation-sensitive PCR assay for specific changes in methylation in CTCF target genes.**

We are in process of conducting these experiments (Months 12-18).

**Task 3. Determine if CTCF expression or function in prostate cancer can predict clinical outcomes. (Months 8-24).**

We are in process of analyzing tissue microarrays using the Vectra™ (Caliper Life Sciences, Inc, Hopkinton, MA) instrument for extracting proteomic and morphometric information. This system accurately measures protein expressions in distinct tissue compartment of interest. We would conduct these experiments between month 12-24 timepoint. We have optimized staining for CTCF, Boris and CHD8 and will contain slides to directly compare expression in both epithelial and stromal compartments.

**KEY RESEARCH ACCOMPLISHMENTS:**

- We have established stable cell lines containing inducible CTCF shRNA in PPC-1, LNCaP, non-tumorigenic HPV16 E6 and E7 prostate cell lines and in 293T cell lines.
- We show that we get effective silencing of CTCF protein in these cell lines expressing CTCF shRNA after inducing the shRNA expression with doxycycline for 3-5 days.
- CTCF knockdown resulted in decrease in proliferation.

**REPORTABLE OUTCOMES:**

- Pending

**Manuscripts:**

- Pending

## **CONCLUSION:**

We have discovered that CTCF knockdown in prostate cancer cells (LNCaP, PPC-1) causes a decrease in proliferation. These results are in contrast to a proposed role for CTCF as a tumor suppressor gene where CTCF knockdown would lead to increase in cell proliferation (8). Alternatively, there is also a published report of CTCF knockout mice where thymus cells isolated from these mice exhibit cell cycle arrest (9). Our ongoing experiments of CTCF knockdown in normal HPECs, non-tumorigenic HPV16 E6 and E7 cells will help us better understand the role of CTCF in cell cycle progression. Our future experiments would examine local and global methylation changes in response to CTCF ablation in prostate cells. Finally, we would examine the CTCF expression using tissue microarrays and correlate CTCF expression with various clinical parameters.

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