AWARD NUMBER: W81XWH-14-1-0280

TITLE: Cells of Origin of Epithelial Ovarian Cancers

PRINCIPAL INVESTIGATOR: Zhe Li, PhD

CONTRACTING ORGANIZATION: THE BRIGHAM AND WOMEN'S HOSPITAL, INC.
BOSTON, MA 02115

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Fort Detrick, Maryland 21702-5012

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Epithelial ovarian cancer (EOC) is the most lethal malignancy of the female reproductive system, largely due to the fact that most EOCs are diagnosed only after the cancer has metastasized into the peritoneal space of the patient. Thus, a better understanding of the cellular origin and early stages of EOC will have important implications for prevention and early diagnosis of EOC. Both ovarian surface epithelial and fallopian tubal epithelial cells have been proposed as cells of origin of EOC. To determine cellular origin of EOC and to test whether different oncogenic events contribute to different subtypes of EOC, we utilize Cre-expressing adenovirus under the control of a keratin 8 (K8) promoter (Ad-K8-Cre) to initiate oncogenic events in K8+ ovarian surface epithelial and fallopian tubal epithelial cells, coupled with lineage-tracing. By immunostaining and flow cytometric analyses, we found that K8 is expressed in ovarian surface epithelial and fallopian tubal epithelial cells and its expression correlates with that of Lgr5, a marker of ovary and tubal epithelial stem/progenitor cells. Intrabursal injection of Ad-K8-Cre to Rosa26-STOP-YFP reporter mice leads to genetic marking of two subpopulations of epithelial cells in both ovary and fallopian tube, which overlap with the Lgr5+ subpopulations. Currently our effort focuses on targeting the K8+ population for EOC initiation.
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1. INTRODUCTION:

Epithelial ovarian cancer (EOC) is the most lethal malignancy of the female reproductive system, largely due to the fact that most EOCs are diagnosed only after the cancer has metastasized into the peritoneal space of the patient (Quartuccio et al., 2013). Thus, in order to improve the outcome of EOC patients, one of the major challenges is to understand the early stage of the disease, e.g., what is the cellular origin of EOC; what is the precursor lesion and what are the early molecular changes in the precursor lesion? A better understanding of these questions will lead to novel strategies for prevention and early diagnosis of EOC, as well as for new approaches to treat EOC. Both ovarian surface epithelium (OSE) and fimbrial epithelium of the fallopian tubes [i.e., part of the fallopian tubal epithelium (FTE)] have been proposed as cells of origin of EOCs (Chene et al., 2013; Ng and Barker, 2015). Recently, a population of Lgr5+ cells was identified in both OSE and FTE and importantly, adult Lgr5+ cells maintain OSE homeostasis and ovulatory regenerative repair in vivo, and there was also indirect evidence to support that these Lgr5+ cells might serve as cells of origin of EOC (Flesken-Nikitin et al., 2013; Ng et al., 2014). However, direct evidence to support Lgr5+ cells as cells of origin of EOC is still lacking and whether these cells give rise to different subtypes of EOCs upon transformation by different oncogenic events remains elusive. Mouse models are instrumental in addressing these questions. Current EOC mouse models often rely on intrabursal injection of Cre-expressing adenovirus (Ad-Cre) under the control of the constitutive CMV promoter (Ad-CMV-Cre) to mice carrying conditional knockout alleles for tumor suppressors (e.g., Trp53, Rb1) (Flesken-Nikitin et al., 2013). However in this approach, both stem cells and non-stem cells in OSE and FTE, as well as bursal cells, could be targeted. It is of great interest to target Cre expression specifically to the stem cell-subset of OSE or to fimbrial epithelium (e.g., Lgr5+ cells) to initiate EOC development. In addition, by targeting different oncogenic events to distinct subsets of epithelial cells and by lineage tracing, one can also characterize the early changes during their transformation, thus providing a unique opportunity to study the early stage of EOC development; one can also determine whether the same subset of epithelial cells gives rise to different subtypes of EOCs, when transformed by different oncogenic events. The purpose of this project is to initiate a pilot study to target Cre-expression to different subsets of OSE cells (e.g., the Lgr5+ stem cell-subset of OSE) or to OSE vs. FTE cells, by using Ad-Cre under the control of cell type-specific promoters, to reveal their in vivo fates by lineage tracing, and to study EOC development from them, and to determine whether different types of EOCs may be developed from the same target cell population under the influence of different oncogenic events.

2. KEYWORDS: Epithelial ovarian cancer, cellular origin, cell of origin, ovarian surface epithelium, fallopian tubal epithelium, Lgr5, stem cell, lineage tracing, adenovirus, Ad-Cre.

3. ACCOMPLISHMENTS:
   o What were the major goals of the project?

There are two major goals (Specific Aims) of this project:
   1) Reveal the in vivo fates of different subsets of OSE cells targeted by cell type-specific Ad-Cre viruses.
   2) Determine the phenotypes of EOCs developed from distinct subsets of OSE cells with different combinations of oncogenic events.

   o What was accomplished under these goals?

During the first year of this project, we successfully recruited a new postdoctoral fellow with extensive experience in ovary research (ovarian physiology, oogonial stem cells) to work on this project. We also
obtained approval of our animal protocol for this project both from our local IACUC and from ACURO. Specific research activities and key results are listed below (based on SOW):

<table>
<thead>
<tr>
<th>Specific Aim 1: Reveal the in vivo fates of different subsets of OSE cells targeted by cell type-specific Ad-Cre viruses</th>
<th>Months</th>
<th>BWH</th>
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</thead>
<tbody>
<tr>
<td><strong>Major Task 1: Establish the Ad-Cre intrabursal injection approach</strong></td>
<td></td>
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</tr>
<tr>
<td>Subtask 1: Lab personnel recruitment and training</td>
<td>1-6</td>
<td>Dr. Li</td>
</tr>
<tr>
<td>Progress: accomplished</td>
<td></td>
<td></td>
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<tr>
<td>Subtask 2: Amend our animal protocol for the Ad-Cre intrabursal injection procedure and apply for approval from our IACUC</td>
<td>1-2</td>
<td>Dr. Li</td>
</tr>
<tr>
<td>Progress: accomplished</td>
<td></td>
<td></td>
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<tr>
<td>Subtask 3: Submit documents for ACURO approvals</td>
<td>3-6</td>
<td>Dr. Li</td>
</tr>
<tr>
<td>Progress: accomplished</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Milestones Achieved: Obtain IACUC and ACURO approvals</strong></td>
<td></td>
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<tr>
<td>Progress: achieved</td>
<td>6</td>
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**Major Task 2: Test several cell type-specific Ad-Cre viruses by intrabursal injection**

Subtask 1: Validate expression of select marker genes for OSE stem or non-stem cells by immunostaining or quantitative RT-PCR

Progress: Our original proposal was based on a previous paper reporting that OSE stem cells, defined largely based on a Wnt signaling marker, Lgr5 (Lgr5+ cells), were restricted to the hilum region of OSE (Flesken-Nikitin et al., 2013). Later, another study using the same \textit{Lgr5-EGFP-IRES-creERT2} knockin mouse line showed that Lgr5+ cells were more widely distributed in the OSE and were also found in FTE (e.g., the fimbrial epithelium of the fallopian tube) (Ng et al., 2014) (also confirmed by us, see Figure 3). Thus, our original plan to validate expression of marker genes in the OSE stem cells (hilum region) vs. non-stem cells (other region in the OSE) needed to be revised. In order to achieve this, we first used flow cytometry (FACS analysis) to try to delineate different cell subpopulations in the ovary (and fallopian tube), based on cell surface marker expression, in combination with the GFP signal from the \textit{Lgr5-EGFP-IRES-creERT2} knockin allele (originally Lgr5+ cells were detected based on this EGFP reporter). After optimizing the cell dissociation protocol, we prepared single cell suspensions from the ovary and fallopian tube and subjected them to FACS analysis. Based on CD24 or EpCAM and CD29 staining [after gating for lineage-negative (Lin’) cells; lineage markers include CD31 endothelial cells, CD45 hematopoietic cells, CD34 stromal cells, and Ter119 erythroid cells], we found that cells from ovary or fallopian tube could be separated into several subpopulations (Figure 1). To determine where Lgr5’ stem/progenitor cells are present in this FACS plot, we analyzed GFP’ (thus Lgr5’ cells) in ovaries and fallopian tubes from \textit{Lgr5-EGFP-IRES-creERT2} knockin female mice. We found that: 1) ovary contains a lot more Lgr5’ cells than fallopian tube; 2) in both ovary and fallopian tube, Lgr5’ cells are mainly Lin’;EpCAM (or CD24)\textsuperscript{low};CD29’ cells and Lin’;EpCAM (or CD24);
CD29\textsuperscript{+} cells (Figure 1); CD24 has a similar staining pattern to EpCAM, but since
EpCAM staining allows a better separation of cell subpopulations, we mainly used
EpCAM/CD29 co-staining in most of our FACS studies. In some earlier experiments,
we sorted cells from ovaries and fallopian tubes (based on CD24 and CD29) into 4
subpopulations (i.e., CD24 or CD29 single positives, double positive, double negative)
and measured expression levels of several marker genes, including K8, K5, K14 and
Pax8 (FTE cell marker); although the data was a bit noisy due to low cell numbers
(after FACS sorting), we found that Pax8 was expressed in several subpopulations
(e.g., double negative, double positive, CD29-single positive) from fallopian tubes, but
not in those from ovaries; whereas K8 was expressed in both ovaries and fallopian
tubes; however, we could barely detect expression of K5 and K14. To further
determine expression patterns of keratin markers, K8, K14 and K5, in OSE cells and
FTE cells, we performed co-immunofluorescence (co-IF) staining. We found that K8
is highly expressed in both OSE and FTE (in particular in the latter, Figure 2). We did
not observe robust staining of K14 and K5 in these two types of epithelia (Figure 2).
By co-IF staining, we found that K8 is co-expressed with Lgr5 (based on GFP staining
in the Lgr5-EGFP-IRES-creERT2 knockin female mice) in both OSE and FTE,
although there is a trend for K8\textsuperscript{high} cells being Lgr5\textsuperscript{low} and vice versa (Figure 3A-B).
Please note here we largely focused on validating expression patterns of keratin
markers, as their expression patterns are usually epithelial cell type-specific and their
genes have small control regions (i.e., promoters typically less than 3kb), thus
their promoters are particularly suitable for driving Cre expression in adenoviral vector
(which has a packaging capacity of less than 7kb). Other non-keratin markers, which
typically have larger and less characterized promoters, are less practical for our Cre-
targeting purpose here.

Subtask 2: Test existing cell type-specific Ad-Cre viruses (e.g., Ad-K8-Cre, Ad-
K14-Cre) by intrabursal injection in R26Y females

Progress: Since only K8 exhibits robust staining in OSE and FTE (Figures 2-3), we
focused on testing this Ad-Cre virus first. We successfully established the Ad-Cre
intrabursal injection procedure to ovary and fallopian tube, and obtained approval to
perform this procedure in mice from our animal facility. By using this surgical
procedure, we injected Ad-K8-Cre virus to 8-week old Rosa26-STOP-YFP (R26Y)
conditional reporter females and found that Ad-K8-Cre could turn on YFP expression
in OSE cells (Figure 4A, IF staining). By FACS analysis, we found that cells in ovary
targeted by Ad-K8-Cre (i.e., YFP\textsuperscript{+} cells) included a large population of Lin\textsuperscript{+};
EpCAM\textsuperscript{+};CD29\textsuperscript{+} cells and a smaller population of Lin\textsuperscript{−};EpCAM\textsuperscript{+};CD29\textsuperscript{+} cells (Figure
4B), the two populations of cells that are also targeted by Lgr5 (Figure 1). In fallopian
tube of the injected females, we also observed YFP-marked cells and these YFP\textsuperscript{+} cells
represented Lin\textsuperscript{+};EpCAM\textsuperscript{+};CD29\textsuperscript{+} and Lin\textsuperscript{−};EpCAM\textsuperscript{+};CD29\textsuperscript{+} cells as well (Figure
4B). Although we did not detect robust K5 expression in OSE or FTE, previously it
was shown that the K5 promoter was active in OSE cells in mice [shown as TVA
expression driven by the K5 promoter (Orsulic et al., 2002)], therefore we also
decided to test K5-Cre expressing adenovirus (Ad-K5-Cre) by intrabursal injection.
Ad-K5-Cre is available from the University of Iowa Gene Transfer Core; unfortunately
the investigator who originally generated and deposited this Ad-Cre did not authorize
the release of it (to the general research community) until recently. We are now
getting this Ad-Cre and will test it by intrabursal injection as well.
### Specific Aim 2: Determine the phenotypes of EOCs developed from distinct subsets of OSE cells with different combinations of oncogenic events

#### Major Task 3: Induce EOC development from female mice with different combinations of oncogenic events

<table>
<thead>
<tr>
<th>Subtask 1: Purchase additional conditional mouse strains from JAX</th>
</tr>
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<tbody>
<tr>
<td>[\text{LSL-Kras}^{G12D^{+/+}}] (JAX strain # 008179) or [\text{Apc}^{Jax}] (JAX strain # 009045), [\text{Rosa26}\text{Pik3ca}{^*H1047R^{+/+}}] (JAX strain # 016977), and [\text{Dicer}^{Jax}] (JAX strain # 006366 or 012284)</td>
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<tr>
<td>Progress: accomplished</td>
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<td>6-7</td>
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<tr>
<th>Subtask 2: Breed various conditional mouse lines to generate and expand various double transgenic females</th>
</tr>
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<tr>
<td>[\text{p53}^{lox/lox}\text{;Rb}^{lox/lox}\text{;Brcal}^{lox/lox}\text{;Pten}^{lox/lox}\text{;LSL-Kras}^{G12D^{+/+}}\text{ or Pten}^{lox/lox}\text{;Apc}^{lox/lox}\text{;Pten}^{lox/lox}\text{;Rosa26}\text{Pik3ca}{^*H1047R^{+/+}}\text{, and Pten}^{lox/lox}\text{;Dicer}^{lox/lox}]</td>
</tr>
<tr>
<td>Progress: accomplished; various breedings are being continued</td>
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<td>7-24</td>
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<table>
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<tr>
<th>Subtask 3: Induce EOC development in various transgenic female mice by</th>
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<td>13-24</td>
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</table>
intrabursal injection of cell type-specific Ad-Cre

Progress: in progress. So far we have already started to inject Ad-K8-Cre to various allele combos, including $Pten^{lox/lox};Rosa26^{PkoE3a^cH1047R/+}$, $Pten^{lox/lox};Dicer^{lox/lox}$, $Pten^{lox/lox};LSL-Kras^{G12D/+}$, $p53^{lox/lox};Brca1^{lox/lox}$, and $p53^{lox/lox};Rb1^{lox/lox}$.

Subtask 4: Determine the subtype(s) of EOC developed in double transgenic females upon Ad-Cre injection

Progress: in progress. We are currently monitoring the injected females for potential ovarian cancer development.

Milestone(s) Achieved: Determine how initiating oncogenic events and cells of origin affect the subtype(s) of EOCs; prepare manuscript(s)

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<tr>
<th>Subtask 4</th>
<th>Milestone(s) Achieved</th>
<th>Author</th>
</tr>
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<tbody>
<tr>
<td>Determine the subtype(s) of EOC developed in double transgenic females</td>
<td>Determine how initiating oncogenic events and cells of origin affect the subtype(s) of EOCs; prepare manuscript(s)</td>
<td>Dr. Li</td>
</tr>
</tbody>
</table>

13-24 Dr. Li

**Figure 1.** FACS analysis showing cells in ovary and fallopian tube can be separated into several populations based on EpCAM and CD29 staining, after gating for Lin-DAPI (i.e., lineage-negative live cells). In Lgr5-EGFP-IRES-creERT2 knockin female mice, GFP (Lgr5)$^+$ cells could be detected and could be separated into two subpopulations based on EpCAM and CD29 staining (circles).

**Figure 2.** Co-IF staining of ovary and fallopian tube sections for K8 (red), K5 (top) or K14 (bottom) (green) and nuclei (DAPI, blue) showing OSE and FTE cells express K8, but not K5 or K14, robustly. O: ovary; FT: fallopian tube.
Figure 3. A-B: Co-IF staining for K8 (red), Lgr5 (GFP, green) and nuclei (DAPI, blue) showing K8+ and Lgr5+ cells were largely overlapping in OSE (A-B) and fallopian tube (B, though less obvious due to low number of Lgr5+ cells in fallopian tube).

Figure 4. Intrabursal injection of Ad-K8-Cre to R26Y reporter female mice led to genetic marking of K8+ OSE and fallopian tubal epithelial cells by YFP. A: IF staining showing YFP-marked OSE cells (green, arrows). B: FACS analysis showing YFP-marked OSE and fallopian tubal epithelial cells were localized in two FACS gates based on EpCAM and CD29 staining, similar to those of Lgr5.

Reference:


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

Nothing to Report.

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?

We will perform lineage-tracing experiments to determine the \textit{in vivo} fate of normal K8$^+$ epithelial cells in ovary and fallopian tube targeted by \textit{Ad-K8-Cre} [i.e., cells of origin of EOCs (if any) from experiments in Aim 2]. We are currently performing more intrabursal injection experiments to introduce \textit{Ad-K8-Cre} to \textit{R26Y} reporter females and will analyze the injected females (by FACS and co-IF staining) at various time points after injection (e.g., several days, several weeks, or several months post-injection). We will finish generating double transgenic mice for all the proposed allele combinations and will start to systematically generate final experimental female mice. We will inject \textit{Ad-K8-Cre} (or control \textit{Ad-CMV-Cre}) to these experimental females and monitor any potential EOC development in them. If any of such females develop EOC, we will characterize them by histological and co-IF staining analysis, as well as FACS analysis, to determine their histological subtypes and cell populations.

4. IMPACT:

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

By completing this project, we expect to make an impact on ovarian cancer research, management (e.g., early detection) and treatment in several aspects: 1) Reveal whether different oncogenic events dictate the subtype of EOC developed from the same cellular origin; 2) Reveal the relation of EOCs (if any) originated from OSE versus those originated from fallopian tubal epithelium; 3) Reveal key cell events (e.g., dynamics in cell populations) at early stages of EOC development.

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
   - Actual or anticipated problems or delays and actions or plans to resolve them
   - Changes that had a significant impact on expenditures
   - Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
   - Significant changes in use or care of human subjects
   - Significant changes in use or care of vertebrate animals.
   - Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS:
   - Publications, conference papers, and presentations
     - Journal publications.
     - Books or other non-periodical, one-time publications.
     - Other publications, conference papers, and presentations.
   - Website(s) or other Internet site(s)
   - Technologies or techniques
   - Inventions, patent applications, and/or licenses
   - Other Products

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS
   - What individuals have worked on the project?

<table>
<thead>
<tr>
<th>Name:</th>
<th>Zhe Li, PhD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Project Role:</td>
<td>PI</td>
</tr>
<tr>
<td>Researcher Identifier (e.g. ORCID ID):</td>
<td>n/a</td>
</tr>
<tr>
<td>Nearest person month worked:</td>
<td>3</td>
</tr>
<tr>
<td>Contribution to Project:</td>
<td>Dr. Li has supervised the overall design and implementation of the experiments and has been involved in data analysis</td>
</tr>
</tbody>
</table>
Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Since the award of this grant, the active support of the PI, Dr. Zhe Li, has changed: the DOD/PCRP award (PC100704 or W81XWH-11-1-0329, Genome-Wide Search of Oncogenic Pathways Cooperating with ETS Fusions in Prostate Cancer), the DF/HCC sponsored A. David Mazzone Research Awards Program award (2012_PD_156, Castration-Resistant Luminal Cells in the Prostate), and the Harvard Stem Cell Institute award (DP-0137-13-00, Pathway-based quantitative clonal analysis for studying cancer stem cells in vivo) have ended. Several new grants have been awarded and are listed below:

- **R21 DE024395 (Li)**  
  07/01/2014 - 06/30/2016  
  1.5 calendar months  
  NIH/NIDCR  
  Contact: Program Official (PO): VENKATACHALAM, SUNDARESAN

Lineage tracing and clonal analysis of oral cancer initiating cells

The goal of this project is to study cancer stem cells/cancer initiating cells in oral squamous cell carcinomas by a novel pathway-based lineage tracing approach in a murine model.
Specific aims:
1. Determine whether oral cancer cells genetically marked based on their activities for stem cell-related pathways exhibit cancer stem cell properties in vivo by quantitative clonal analysis
2. Determine how oral cancer initiating cells defined based on their activity for stem cell-related pathways respond to therapeutic interventions in vivo at the clonal level
Role: PI
OVERLAP: No
W81XWH-15-1-0100 (Li) 05/01/2015 - 04/30/2018 1.5 calendar months
DOD/BCRP
Contact: Ashley Schneekloth, Ph.D., Science Officer, Ripple Effect Communications in support of Congressionally Directed Medical Research Programs (CDMRP)
1077 Patchel Street, Ft. Detrick, MD 21702

Context-Dependent Roles of LSD1 in Mammary Epithelial Cells and Breast Cancer
The goal of this project is to study context-dependent roles of the epigenetic regulator LSD1 in development of different subsets of mammary epithelial cells and different subtypes of breast cancer.
Specific aims:
1. Define distinct roles of LSD1 in basal and luminal MECs
2. Determine whether LSD1 functions as either a tumor suppressor or an oncoprotein in a breast cancer subtype and/or stage-specific manner
Role: PI
OVERLAP: No
W81XWH-15-1-0546 (Li) 09/15/2015 - 09/14/2018 1.5 calendar months
DOD/PCRP
Contact: Ram Arudchandran, Ph.D., Health Science Program Manager
Science Officer of Prostate Cancer Research Programs
Congressionally Directed Medical Research Program (CDMRP)
U.S. Army Medical Research and Material Command (USAMRMC)
1053 Patchel Street, Fort Detrick, MD 21702-5012

Castration-resistant luminal cells active for Wnt signaling as cells of origin of castration-resistant prostate cancer
The goal of this project is to characterize the intrinsic properties of castration-resistant prostate luminal cells active for Wnt signaling and determine their role in prostate cancer in murine models.
Specific aims:
1. Characterize the subpopulation of castration-resistant prostate luminal cells active for Wnt signaling
2. Test whether castration-resistant prostate cells active for Wnt signaling can serve as cells of origin of more aggressive prostate cancer and whether they play a key role in maintaining castration-resistant prostate cancer
Role: PI
OVERLAP: No

What other organizations were involved as partners?
Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS
   o COLLABORATIVE AWARDS:
   o QUAD CHARTS:

Not applicable.

9. APPENDICES:

Nothing to report.