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TITLE: TRANSCRIPTIONAL MODULATION OF TUMOR-ASSOCIATED MACROPHAGES TO FACILITATE PROSTATE CANCER IMMUNOTHERAPY

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TRANSCRIPTIONAL MODULATION OF TUMOR-ASSOCIATED MACROPHAGES TO FACILITATE PROSTATE CANCER IMMUNOTHERAPY

Prostate cancer (PCa) contains abundant tumor-associated macrophages (TAMs), with increased TAM M2 polarization correlating with disease stage, emergence of castration-resistance, and worse prognosis. We hypothesize that targeting TAM transcription factors that mediate their M2 polarization, reprogramming them into the pro-inflammatory M1 state, will provide a novel approach to PCa therapy, and we seek to assess whether adoptive transfer monocytes lacking a key TAM transcription factor shows therapeutic utility, alone or with checkpoint inhibition. During the current reporting year we found that a murine PCa line grows slower in mice lacking NF-kB p50 compared with wild-type controls and in KLF4(f/f);Lys-Cre mice vs KLF4(f/f) controls, with increased TAM M1 polarization, particularly in p50-/− hosts, and with increased number and activation of tumor T cells, predominantly CD4 T cells in p50-/− vs wild-type and CD8 T cells in KLF4(f/f);Lys-Cre vs KLF4(f/f) tumor recipients. We also demonstrated that expansion of marrow progenitors in SCF/FL/TPO for 6 days, culture in M-CSF for 1 day, and adoptive transfer allows PCa tumor localization in preference to normal organs. And we find that anti-PD-1 is active in our tumor model. Thus, we have identified two transcription factors whose down-regulation in PCa TAMs contributes to tumor control and have begun to optimize a novel immunotherapy including adoptive transfer of gene modified myeloid cells.
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1. INTRODUCTION

Prostate cancer (PCa) contains abundant tumor-associated macrophages (TAMs), with their increased content and percentage of M2 polarization correlating with increased disease stage, emergence of castration-resistant disease, and worse prognosis. We hypothesize that targeting TAM transcription factors that mediate their M2 polarization, reprogramming them into the pro-inflammatory M1 state, would provide a novel approach to prostate cancer therapy. This project first seeks to determine whether syngeneic murine PCa cells, unselected or castration resistant, grow more slowly in mice lacking NF-κB p50 (p50), KLF4, or PU.1 in their myeloid cells, correlated with increased TAM M1 polarization and increased activation of tumor-infiltrating lymphocytes. We then seek to assess whether adoptive transfer or murine monocytes lacking p50, KLF4, or PU.1 slows PCa growth, alone or in synergy with CSF1R small molecule tyrosine kinase inhibitor (TKI) or antibody (Ab) targeting or with anti-PD-1 or anti-CTLA4 T cell checkpoint inhibitory antibodies.

2. KEYWORDS

Prostate cancer, immunotherapy, tumor-associated macrophages, NF-κB p50, KLF4

3. ACCOMPLISHMENTS

Major goals of the project

Task 1: Assess prostate cancer growth in WT versus p50-/-, KLF4(f/f);Lys-Cre, and PU.1(kd/kd) B6 mice
Subtask 1 - Obtain ACURO approval (mos 1-3), completed prior to 9/01/2016 start date
Subtask 2 - Assess PCa growth in WT vs mutant recipients (mos 4-12), completed 6/01/17
Subtask 3 - Assess castration-resistant growth in WT vs mutant recipients (mos 6-24), 25% complete
Subtask 4 - Assess metastatic growth in WT vs mutant recipients (mos 6-24), 0% complete

Task 2: TAM polarization and T cell activation in WT vs p50-/-, KLF4(f/f);Lys-Cre, and PU.1(kd/kd) mice
Subtask 1 - Obtain ACURO approval (mos 3-5), completed prior to 9/01/2016 start date
Subtask 2 – Assess TAM polarization and T cell numbers and activation (mos 6-24), 75% complete

Task 3: Assess the effect of adoptively transferred M2-defective M1 macrophages on prostate cancer
Subtask 1 - Obtain ACURO approval (mos 2-5), completed prior to 9/01/2016 start date
Subtask 2 - Determine effects of adoptively transferred monocytes (mos 6-24), 25% complete
Subtask 3 - Determine whether infused monocytes reach the tumor (mos 6-24), 50% complete
Subtask 4 - Determine how infused monocytes alter tumor TAMs and T cells (mos 6-24), 0% complete

Task 4: Assess synergy between CSF1R blockade and adoptive transfer of M2-defective M1 monocytes
Subtask 1 - Obtain ACURO approval (mos 8-11), completed prior to 9/01/2016 start date
Subtask 2 - Determine the effect of MCSFR TKI on prostate cancer growth (mos 12-36), 0% complete
Subtask 3 - Determine synergy between MCSFR TKI and monocytes (mos 18-36), 0% complete
Subtask 4 - Determine the effect of MCSFR TKI and monocytes on tumor TAMs/T cells (mos 18-36), 0%
Subtask 5 - Determine synergy between MCSFR Ab and monocytes (mos 18-36), 10% complete
Subtask 6 - Determine the effect of MCSFR Ab and monocytes on tumor TAMs/T cells (mos 18-36), 0%

Task 5: Assess synergy between checkpoint blockade and adoptive transfer of M2-defective M1 monocytes
Subtask 1 - Obtain ACURO approval (mos 8-11), completed prior to 9/01/2016 start date
Subtask 2 - Determine synergy between PD-1 Ab and monocytes on tumor growth (mos 12-36), 25%
Subtask 3 - Determine synergy between CTLA4 Ab and monocytes on tumor growth (mos 12-36), 0%
Subtask 4 - Determine synergy between PD-1/CTLA4 Ab and monocytes on tumor T cells (mos 12-36), 0%
Accomplishments under these goals

**Task 1/Subtask 2 – Assess prostate cancer growth in WT versus mutant mice**

B6 Hi-Myc SQ tumors were dissociated with collagenase, hyaluronidase, Dispase, and DNaseI, followed by enumeration of viable cells with Trypan blue dye. 2E6 viable cells were inoculated SQ into the shaved flanks of WT or p50/- B6 male recipients anesthetized with isoflurane. Tumor length (L) and width (W) were measured with a caliper twice weekly, with \( V = L \times W^2 / 2 \) used to estimate tumor volumes. Tumors are too small to measure prior to day 20. Volume measurements obtained thereafter in WT and p50/- recipients are shown, with slower growth in p50/- recipients evident (Fig. 1A). When volumes on day 28 are compared, the tumors in p50/- hosts are on average 4-fold smaller (Fig. 1B). As a more accurate means to compare growth, these data were replotted on a log scale, with best-fit lines according to the equation \( V = V_0 \times e^{bt} \) shown (Fig. 1C). The average slopes of these logarithmic PCa growth curves is ~2-fold lower in p50/- recipients (Fig. 1D).

An analogous experiment was carried out using KLF4(f/f) vs. KLF(f/f);Lys-Cre recipients. Hi-Myc PCa tumor growth was markedly slower in mice lacking macrophage KLF4 (Fig. 2A), with ~6-fold reduced tumor volume, on average, on D29 (Fig. 2B). After logarithmic transformation, the growth curve slopes were on average ~2-fold lower in KLF4(f/f);Lys-Cre compared with KLF4(f/f) recipients (Fig. 1C, D).

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**Fig. 1**

<table>
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<tr>
<td><strong>Tumor volume (mm³)</strong></td>
<td><strong>Tumor volume, D28</strong></td>
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<tr>
<td>Days post-implantation</td>
<td><strong>WT (n=8)</strong></td>
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![Tumor volume graph](image1)

**Fig. 2**

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<td><strong>Tumor volume (mm³)</strong></td>
<td><strong>Tumor volume, D29</strong></td>
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<tr>
<td>Days post-implantation</td>
<td><strong>KLF4ff (n=7)</strong></td>
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![Tumor volume graph](image2)
**Task 1/Subtask 3 – Assess castration-resistant prostate cancer growth in WT versus mutant mice**

B6 Hi-Myc PCa tumor-bearing KLF4(f/f) and KLF4(f/f);Lys-Cre male mice were subjected to orchiectomy. These androgen-dependent tumors regressed to an undetectable size over the next 16–42 days, and castration-resistant tumors emerged in two KLF(f/f) mice and one KLF4(f/f);Lys-Cre mouse (Fig. 3). These tumors reached 400 mm³ on D125 in the KLF4(f/f) recipients but not until D160 in the KLF4(f/f);Lys-Cre host, suggesting that absence of KLF4 in tumor macrophages slows growth of castration-resistant prostate cancer (CRPC). Given the low penetrance of formation of castration-resistant tumor (3 of 8) and the long time period required for their emergence (3–4 months), we sought to passage one of these CRPCs in wild-type hosts for use in future experiments – this was successful, with tumors now uniformly evident by D30.

**Task 2/Subtask 2 – Assess tumor TAM polarization and T cell numbers and activation in WT vs mutant hosts**

Hi-Myc PCa tumors growing in WT vs p50−/− or KLF4(f/f) vs KLF4(f/f);Lys-Cre hosts were dissociated and analyzed by FACS for myeloid surface markers (n=3). Total myeloid cells, CD45+CD11b+, and TAMs, F4/80+ cells in the myeloid gate, were increased in the absence of p50 or KLF4 (Fig. 4A). TAMs were assessed for mannose receptor (MR, M1 marker), MHCII, and PD-L1 (M2 markers). MR was reduced and both MHCII and PD-L1 were increased in the absence of p50−/−, and PD-L1 was increased in the absence of KLF4 (Fig. 4B). Tumor CD11b+ cells were isolated using Ab-coated beads, and myeloid RNA was then assessed for several M1 and M2 markers by qRT-PCR (n=4). Several M1 markers were elevated and M2 markers reduced in the absence of p50 (Fig. 4C). In contrast, tumor myeloid cell M1 and M2 markers were unaffected by absence of KLF4 (not shown); therefore, we in addition subjected RNAs from CD11b+ cells within PCa tumors forming in KLF4(f/f) vs KLF4(f/f);Lys-Cre mice to microarray global gene expression analysis (n=2). Pathway analysis indicated increased expression of RNAs associated with phagocytosis and with LPS Toll-like receptor signaling (Fig. 5). TLR signaling can increase macrophage phagocytosis, which in turn can slow tumor growth.
Hi-Myc PCa tumor-infiltrating T cells were also analyzed. Total tumor CD3+ T cells increased in 3.5-fold in p50/- and 2-fold in KLF4(f/f);Lys-Cre hosts vs controls; this increase was completely accounted for by CD4 T cells in p50/- recipients and by CD8 T cells in KLF4(f/f);Lys-Cre recipients (Fig. 6A,B). In addition, the numbers of activated tumor CD4 T cells, enumerated as those expressing intracellular IFNγ or surface CD69, was dramatically increased in p50/- vs WT hosts, and the number of activated tumor CD8 T cells was increased approximately 5-fold in KLF4(f/f);Lys-Cre vs KLF4(f/f) tumor recipients (Fig. 6C, D). CD25+Foxp3+ Tregs were unchanged in the absence of KLF4 (not shown).

**Fig. 5**

Genes increased >1.4-fold in PCa from KLF4(f/f);Lys-Cre vs. KLF4(f/f) hosts.

**Task 3/Subtask 2 - Determine effects of adoptively transferred monocytes**

As a first step to studying effects of adoptively transferred monocytes on PCa tumor growth, we sought to optimize in vitro monocyte generation. Marrow cells were subjected to lineage-depletion using a cocktail of Abs that removes mature monocytes, granulocyte, lymphocytes, and erythroblasts. These were then placed in IMDM with 10% FBS and TPO/SCF/FL, cytokines expected to maintain myeloid progenitor immaturity. After 6 days, WT cells expanded 12-fold and p50/- cells 15-fold, with a blast-like morphology (Fig. 7A, upper left). Cells were then transferred to IL3/IL6/SCF, GM-CSF, or M-CSF/SCF for 2 days. IL3/IL6/SCF gave neutrophils and monocytes, whereas GM-CSF or M-CSF/SCF predominantly generated monocytes, with those in GM-CSF appearing more activated (Fig. 7A, upper right and lower).

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### Table: Genes Increased >1.4-fold in PCa

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<td>CCR7</td>
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<td>Gas6</td>
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<td>Mge8</td>
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<tr>
<td>Siglec1</td>
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### Table: Genes Increased >1.4-fold in PCa

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<td>Response to LPS</td>
</tr>
<tr>
<td>S100a8</td>
<td>1.89</td>
<td>Response to LPS</td>
</tr>
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<tr>
<td>Marco</td>
<td>1.98</td>
<td>Response to LPS</td>
</tr>
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</table>
We then compared cells expanded in TPO/SCF/FL for 6d followed by M-CSF or GM-CSF for 1-3 days, for tumor localization using CD45.1+ monocytes infused into CD45.2+ tumor-bearing recipients, finding little localization of GM-CSF exposed cells but that ~8% of tumor CD11b+ myeloid cells derive from infused monocytes exposed to M-CSF for 1 or 2 days, with those exposed to M-CSF for 3 days being much less effective (not shown). We therefore will use M-CSF 1 day in our studies (and not M-CSF for 2 days since these at times might behave poorly as did those exposed for 3 days).

Task 3/Subtask 3 - Determine whether infused monocytes reach prostate cancer tumors
To evaluate monocyte tumor localization vs localization to other organs, Hi-Myc PCa cells were subcutaneously implanted, followed by 5-FU injection on d27 (to reduce marrow monocyte competition, which we find increases infused monocyte tumor localization ~3-fold), tail vein infusion of 10E6 expanded WT CD45.1+ monocytes from CMV/Actin-Luc mice (Jackson Labs), on d31, and IVIS imaging of mice on d33; data shown for isolated organs are from another experiment (Fig. 7B). Infused monocytes localized to the tumor, with lower amounts in lung and spleen. Tumors were dissociated and subject to FACS analysis on d37; in the example shown, 2.6% of viable tumor cells were CD11b+F4/80+ TAMs, and 2.1% of these were CD45.1+ donor cells (Fig. 7C). Interestingly, infused myeloid cells also contributed to ~2% of tumor CD11c+ dendritic cells (not shown).

Task 4/Subtask 5 - Determine synergy between MCSFR Ab and monocytes
We have begun to evaluate the utility of MCSFR Ab for tumor TAM depletion prior to monocyte infusion. As MCSFR Ab would also target infused monocytes, we isolated serum 2, 6, and 10 days after three injections of MCSFR Ab and used this to stain Ba/F3 cells expressing exogenous MCSFR, followed by goat anti-Ig-FITC secondary Ab (Fig. 8). On average (n=3), 32% of cells stained with day 2, 5% with day 6, and 1% with day 10 serum. These data indicate that monocyte infusions will need to be delayed 5-6 days post-MCSFR Ab.

Task 5/Subtask 2 - Determine synergy between PD-1 Ab and monocytes on tumor growth
KLF4(f/f) and KLF4(f/f);Lys-Cre mice inoculated with Hi-Myc PCa cells either received no treatment (NT) or anti-PD-1 Ab (250 µg/dose) on d10, d13, and d16. Tumor volumes were then monitored (4-5/group). Average tumor volumes on subsequent days and on d24 are shown (Fig. 9). As seen before, absence myeloid KLF4 led to markedly slower tumor growth. Anti-PD-1 Ab exposure of KLF4(f/f) mice also markedly slowed tumor growth. Combining KLF4 deletion with anti-PD-1 Ab led to further slowing of tumor growth.
Opportunities for training and professional development

During the past year, this proposal facilitated laboratory-based training and professional development in the fields of prostate cancer research and immunotherapy for two post-doctoral fellows, David Barakat, Ph.D. and Rahul Suresh, Ph.D. In addition to conducting the above experiments, Drs. Barakat and Suresh attended and presented at weekly laboratory meeting held by Drs. Friedman and Pienta, attended the 2016 meeting of the Society for Immunotherapy and Cancer (SITC) at National Harbor, Maryland, and attended numerous scientific seminars at the Johns Hopkins Comprehensive Cancer Center, including Oncology Grand Rounds, Translational Research Conference, and Journal Club.

Dissemination of research results

Nothing to Report.

Plans during the next reporting period

Task 1  As described above, we have obtained a castration-resistant Hi-Myc PCa line that forms SQ tumors 30d after inoculation into castrated WT mice. We will now inoculate WT vs p50/- and KLF4(f/f) vs KLF4(f/f);Lys-Cre mice with this line and monitor tumor growth. We also will inoculate WT vs p50/- and KLF4(f/f) vs KLF4(f/f);Lys-Cre mice with Hi-Myc PCa cells, and once tumors reach 1.2-1.4 cm will remove this and then assess lung metastases 6 wks later.

Task 2  We will further determine whether absence of KLF4 leads to increased tumor macrophage TLR signaling and phagocytosis. TLR signaling activates BTK kinase to stimulated phagocytosis. We will therefore use Western blotting to assess phosphorylated BTK after exposure of KLF4(f/f) vs KLF4(f/f);Lys-Cre macrophages to TLR inducers such as pIpC. We will also compare the ability of these macrophages to phagocyte dye-labeled Hi-Myc PCa cells.

Task 3  We will continue to optimize localization of infused p50/- or KLF4(f/f);Lys-Cre monocytes to Hi-Myc PCa tumors in WT mice, including use of multiple infusions and pre-exposure of mice to 5-FU or Cytoxan prior to monocyte infusion. We will then compare the effects of WT vs p50/- or KLF4(f/f) vs KLF4(f/f);Lys-Cre monocytes (or no monocytes) on tumor growth, TAM numbers and M1 vs M2 phenotypes, and tumor-infiltrating T cell phenotypes.

Task 4  We have received 600 mg of the MCSFR tyrosine kinase inhibitor PLX3397 from Plexxikon. WT mice inoculated with Hi-Myc PCa 14d earlier will be treated with this agent by oral gavage (50 mg/kg/day x 4 days), alone or with subsequent monocyte infusions as in Task 3, followed by assessment of tumor growth and tumor TAM and T cell phenotypes. As described above, we have demonstrated that MCSFR Ab is cleared from serum after 6 days. We will now also expose mice inoculated with Hi-Myc PCa to MCSFR Ab alone or followed 6d later with monocyte infusions, followed by assessment of tumor growth and tumor TAM and T cell phenotypes.

Task 5  As described, we find that three doses of anti-PD-1 antibody markedly slows Hi-Myc PCa tumor growth. To improve our ability to detect synergy, we will now expose WT vs p50/- or KLF4(f/f) vs KLF4(f/f);Lys-Cre mice to one dose of anti-PD-1 antibody, followed by monitoring of tumor growth. We will also conduct this experiment using anti-CTLA4 antibody alone or combined with anti-PD-1. We then intend to combine the most effective checkpoint inhibitor combination and dosing schedule with infusion of monocytes lacking p50 or KLF4.
4. IMPACT

Impact on the development of the principal discipline(s) of the project
Prostate cancers include normal white blood cells called macrophages that contribute to tumor growth. These tumor-associated macrophages (TAMs) suppress the immune system’s ability to fight prostate cancer. This proposal seeks to alter prostate cancer TAMs so that instead of helping the cancer grow they now help the immune system fight the cancer. Like all cells, TAMs contain genes within their DNA that govern their function. Our results indicate that removal of the KLF4 gene, a possibly also the p50 gene, from prostate cancer TAMs slows prostate cancer growth. This finding has implications for the treatment of prostate cancer, as we can now pursue a therapy in which we target KLF4 or p50 in TAMs.

Impact on other disciplines
In addition to prostate cancer, many other cancer contain TAMs that contribute to tumor growth, including brain, pancreatic, and breast cancers. Our findings are therefore also relevant to these and other cancers as targeting TAM KLF4 or p50 may also be effective, alone or in combination with other therapies, for these cancers.

Impact on technology transfer
Nothing to Report.

Impact on society beyond science and technology
Nothing to Report.

5. CHANGES/PROBLEMS

Changes in approach
Nothing to Report.

Actual or anticipated problems or delays and actions or plans to resolve them
As an alternative to use of Hi-Myc PCa cells, we proposed to attempt to develop a related transplantable line from B6 PTEN(f/f);Pb-Cre mice. Although we could establish such lines in tissue culture these did not form tumors in mice. Therefore, we will continue to focus on use of the Hi-Myc PCa line.

In addition to use of WT vs p50-/- and KLF4(f/f) vs KLF4(f/f):Lys-Cre mice, we proposed to compare PCa growth in WT vs PU.1(kd/kd) mice in Task 1. Due to difficulty with breeding we have not yet been able to generate sufficient numbers of male mice for this experiment. When we cross PU.1(kd/kd) male mice with PU.1(kd/kd) females, no viable pups are obtained. Crossing PU.1(kd/kd) with PU.1(kd/+) mice does yield offspring, although at weaning PU.1(kd/kd) pups represent only about 25% rather than 50% of these litters, and of course only half of these are male. We are attempting to solve this problem by expanding the number of breeding cages. Fortunately, our results this past year do show markedly slower tumor growth in p50-/- compared with WT and in KLF4(f/f):Lys-Cre compared with KLF4(f/f) mice, allowing us to proceed with use of these models for all approved Tasks/Subtasks. We had indicated in Aim 1a that we would prioritize use of p50, KLF4, or PU.1 mutant mice for subsequent experiments, choosing the system with the slowest tumor growth. As absence of TAM p50 or KLF4 slowed tumor growth markedly, we will proceed with both of these promising models.

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
The following poster was presented at the Johns Hopkins University School of Medicine 2017 Sidney Kimmel Comprehensive Cancer Center Fellow Research Day:

Barakat DJ, Simons B, Pienta K, Barberi T, Suresh R, Friedman AD. Transcriptional Regulators of Tumor Associated Macrophage Polarization Influence Lymphocyte Infiltration and Growth of Prostate Cancer.

Websites or other internet sites
Nothing to Report.

Technologies or techniques
Nothing to Report.

Inventions, patents, or licenses
Nothing to Report.

Other products
As described in Section 3, we find that prostate cancer grows significantly slower in mice lacking NF-κB p50 or macrophage KLF4, associated with increased tumor macrophage and T cell activation. These findings provide a meaningful contribution towards understanding gene regulatory mechanisms that allow TAMs to contribute to prostate cancer growth and towards developing of novel therapy approaches designed to modify tumor TAMs.

7. PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

Individuals who worked on the project (effort round up to nearest whole month)

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Change in the active other support of the PI and senior/key personnel

Name: Alan D. Friedman
Changes: closed
U01 HL099775, NIH
28520, Alex’s Lemonade Stand Foundation

Name: Kenneth J. Pienta
Changes: closed
90065394, Harvard/DFCI sub-award

new
90069213, CelSee (Pienta), 6/01/16-08/31/17, 0.24 Cal
90070234, Prostate Cancer Foundation (Pienta), 8/01/16-7/31/18, 0.12 Cal
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1U54 CA210173-01S, NIH (Wirtz), 9/01/16-8/31/17, 0.6 Cal
90071565, Progenics Pharmaceuticals Inc. (Pienta), 11/01/16-6/30/18, 0.12 Cal
90072088, Progenics Pharmaceuticals Inc. (Pienta), 1/01/17-12/30/20, 1.44 Cal

Dr. Pienta’s 0.12 Cal annual effort on this DoD award (contract #W81XWH-16-1-0334) will not change as a result of his new Other Support.

Other organizations involved as partners
Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS
Not applicable.

9. APPENDICES
None.