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PRINCIPAL INVESTIGATOR: Vijayasaradhi Setalyr

CONTRACTING ORGANIZATION: University of Wisconsin System
Madison, WI 53715

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Vijayasaradhi Setalyr

E-Mail: vsetaluri@dermatology.wisc.edu

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14. ABSTRACT

Prostate cancer is the most commonly diagnosed cancer in men. Among African American men, the incidence of prostate cancer is approximately 60% higher and the mortality rate in this population is 2 to 3 times greater compared with European American men. The reasons for this disparity are not completely understood. Current tools in hand to study these differences, such as genetically altered mouse models, are useful for dissecting roles of specific genes and signaling pathways in intact animal, but have limited utility for understanding differences in disease susceptibility in humans.

The overall objective of this application is to model prostate epithelial cells to understand the molecular basis for the disparities in prostate cancer risk between white Caucasian and black African-American men. The specific aims are: 1) to establish conditions that promote differentiation of human neonatal foreskin skin fibroblast-derived iPSC into cells with characteristics of prostate epithelium, 2) identify differences in gene expression and epigenetic signatures between prostate epithelial cells derived from iPSC of Caucasian and African-American foreskin fibroblasts and 3) compare and establish methods to transform differentiated prostate epithelial cells to identify differences in susceptibility to transformation.

15. SUBJECT TERMS

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RPPR format for DOD progress report

The text of the report must include all sections addressed in the table of contents to include the following. **DO** include the bolded section headings, but **DO NOT** include the *italicized* descriptions of section contents in your submitted reports.

1. **INTRODUCTION:**

   Prostate cancer is the most commonly diagnosed cancer in men in Europe and the United States. Numerous studies have indicated genetics to have a major role in the etiology of this disease; as much as 42% of the risk may be explained by heritable factors. Moreover, among African American men, the incidence of prostate cancer is approximately 60% higher and the mortality rate in this population is 2 to 3 times greater compared with European American men The reasons for this disparity are not completely understood. Since no clear patterns were observed for association with dietary factors or life style factors such as physical activity, occupational history, sexual behavior and other health conditions), it is likely that inherent genetic and epigenetic differences, presumably both germ-line and prostate-cell specific, contribute to this disparity in prostate cancer risk. Efforts are ongoing to identify molecular mechanism and common risk alleles for prostate cancer risk using genome-wide association studies. While identification of individuals/population at risk is important, additional in-depth studies are needed to understand the genetic and molecular mechanisms responsible for the differences in susceptibility of prostate epithelial cells to malignant transformation. However, limited access to human prostate tissue prior to onset of age-related or malignant changes has hampered analyses of genetic and epigenetic mechanisms intrinsic to prostate epithelial cells. More recent strategies to study prostate development, maturation and carcinogenesis included differentiation of human embryonic stem cells (hESC) using rodent mesenchyme. Studies using hESC also have many limitations including ongoing ethical debate and the number of available cell lines, especially that represent different genetic ancestry. Induced pluripotent stem cells (iPSC) offer a useful alternative to hESC. For example, recently, in vivo regeneration potential of human iPSC has been documented. The proposed project is aimed to test the hypothesis that differentiation of neonatal foreskin fibroblasts-derived iPSC to prostate epithelial cells is a unique and powerful strategy to investigate the genetic and molecular basis for the disparities in prostate cancer risk among men of different genetic ancestry.

2. **KEYWORDS:**

   Induced Pluripotent cells, Directed differentiation, Prostate Cancer, Disparity in Cancer Risk, African-American

3. **ACCOMPLISHMENTS:**

   - **What were the major goals of the project?**

     - To establish culture conditions that promote differentiation of human neonatal foreskin skin fibroblast-derived iPSC into cells with characteristics of prostate epithelium.
     - Identify differences in gene expression and epigenetic signatures between prostate epithelial cells derived from iPSC of Caucasian and African-American foreskin fibroblasts.
     - Compare and establish methods to transform differentiated prostate epithelial cells to identify differences in susceptibility to transformation

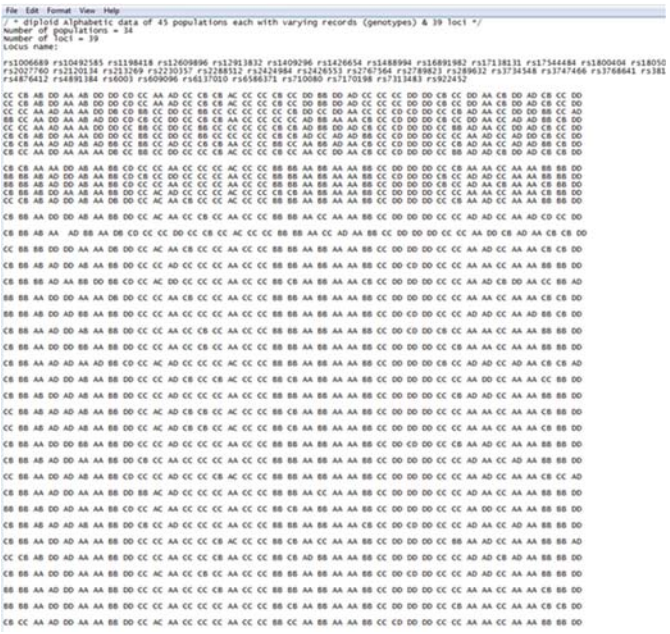
   - **What was accomplished under these goals?**

     - **See below**

In the previous project report, we 1) validated generation of iPSC with normal karyotype (chromosomal analysis of iPSC clones), 2) demonstrated that the iPS cells generated displayed three-germ layer differentiation into ectoderm, mesoderm and endoderm, 3) demonstrated embryoid body formation and characterization of embryoid body and 4) established optimal conditions for prostate organoids differentiation in vitro.

During this reporting period, we identified and validated Caucasian and African-American fibroblasts for iPS generation and prostate differentiation.

Identification of Caucasian and African-American fibroblasts:



Using single nucleotide polymorphisms (SNPs) in 39 Ancestry Informative Markers (AIMs), we analyzed DNA from 45 human primary skin fibroblasts. This analysis involved 45 DNAs X 39 loci= 1,755 samples X 2 = 3,510 alleles. The distribution of samples was as follows: European/Caucasian-American= 34; African-American = 8; Hispanic-American = 2; Asian-American = 1. The columns represents 39 SNP loci and rows represent each individual (n=45).

Fig. 1. Example of data set arrangement for genetic analysis with POPGENE.

Validation of prostate differentiation of iPSC:

To validate prostate differentiation, we generated a plasmid for EGFP reporter expression under PSA promoter (Fig. 2) and generated lentiviruses. To establish the specificity of PSA promoter activity in prostate cells, we transduced iPSC and prostate cancer cell line 22Rv1 and monitored the EGFP expression by fluorescence microscopy (Fig. 3).

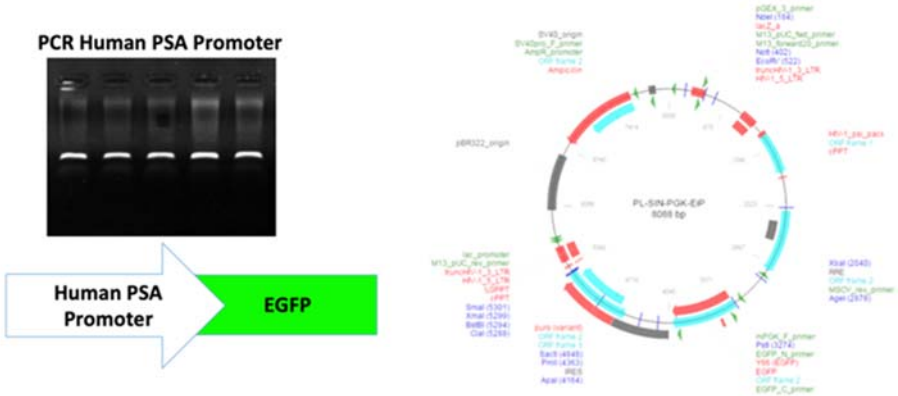


Fig. 2. Cloning of human PSA-Promoter into a lentiviral plasmid. We generated primers and optimized PCR conditions for human PSA-Promoter region (agarose gel). PCR product was inserted into a lentiviral plasmid containing human PGK-promoter EGFP reporter. We replaced the PGK-promoter with PSA-promoter. The plasmid was used for lentiviral production using envelop and packaging plasmids for lentiviral production with HEK293 cells.

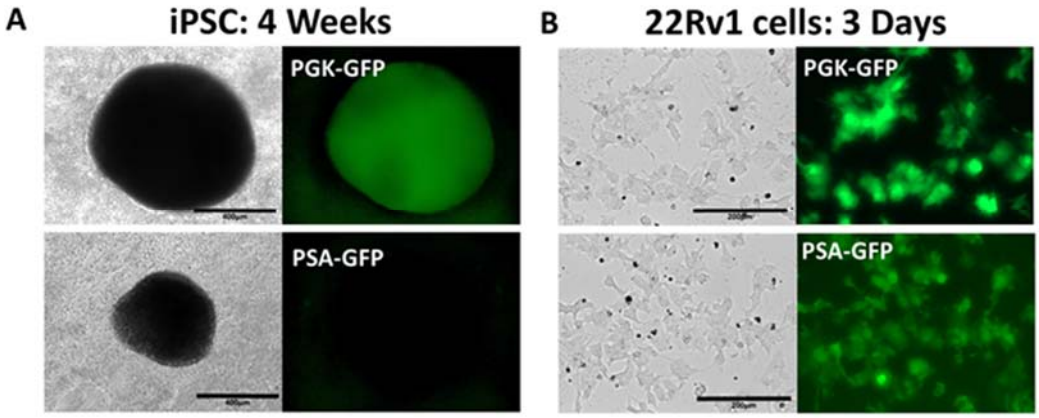


Fig. 3. Expression of PSA-GFP reporter in iPSC and prostate cancer (22Rv1) cells. **A.** Four weeks after of transduction with either housekeeping PGK-GFP (control) or PSA-GFP reporter, iPSCs exhibited expression of PGK-GFP but not PSA-GFP. **B.** In contrast, 22Rv1 prostate cancer cells show expression of both PGK-GFP and PSA-GFP reporters just after three days of transduction. This experiment demonstrate that the PSA-GFP lentiviral reporter is highly specific to prostate cells.

Then, we used PSA-EGFP transduced iPSC to monitor prostate differentiation of iPSC (Fig. 4).

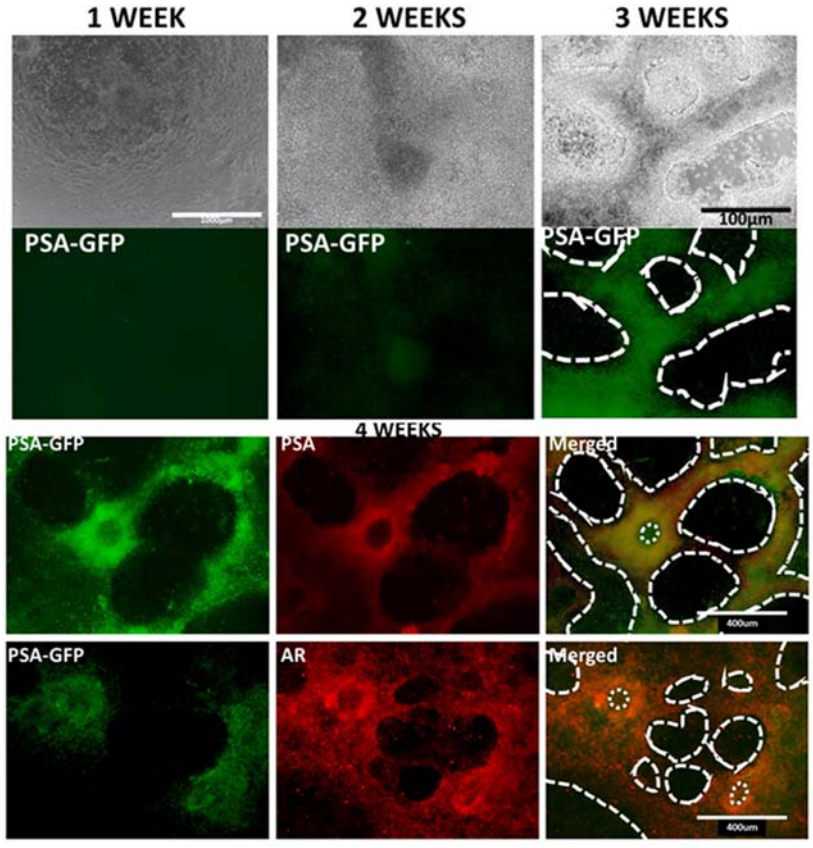


Fig. 4. Time-course expression of PSA-GFP reporter during prostate differentiation of iPSC cells. We transduced iPSC cells with the PSA-GFP reporter one day before prostate differentiation. **(Top)** The first week after prostate differentiation, the reporter did not show GFP expression. After two weeks of differentiation, the reporter showed a weak expression. The third week the reporter showed stronger expression of GFP and it was accompanied by prostate-like morphogenesis such as acinar-like structures. **(Bottom)** After four weeks, the organoids showed the strongest expression and we determined the expression of endogenous PSA and AR by immunofluorescence. Results indicate that as expected, PSA-GFP reporter was highly colocalized and enriched with endogenous PSA expresed by the organoid in areas with acinar-like structures. However, although AR (androgen receptor) co-expression was noted with EGFP, its expression was more widely distributed.

To validate the prostate differentiation of fibroblast-derived iPSC further, we compared the differentiation of fibroblast-derived iPSC with prostate cancer cell line 22Rv1-derived iPSC (Figs. 5 and 6) using the PSA promoter-EGFP reporter.

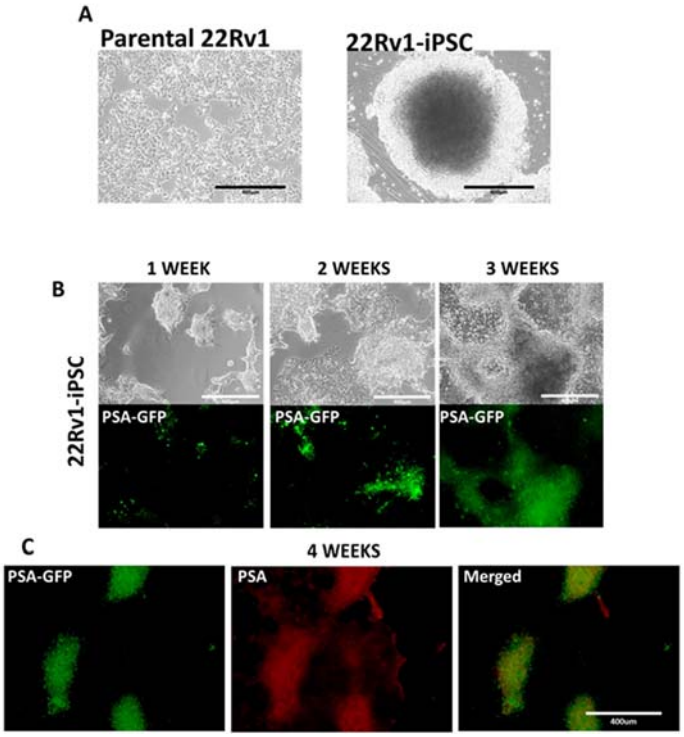


Fig. 5. Prostate organoids from 22Rv1-derived iPSC. We generated iPSC from 22Rv1 prostate cancer cells (A). 22Rv1-iPSC were transduced with PSA-EGFP reporter and subjected to prostate differentiation (B-C). Results showed that 22Rv1-derived iPSC did not form prostate organoids efficiently as fibroblasts-derived iPSC. The organoids did not form acinar-like structures or prostate-related morphogenesis after 1-3 weeks of differentiation (B). At the fourth week, we determined the expression of endogenous PSA (C). Results showed that although PSA-GFP reporter colocalized with endogenous PSA expression, the organoids did not form the acinar-like structures as observed in fibroblasts-derived iPSC.

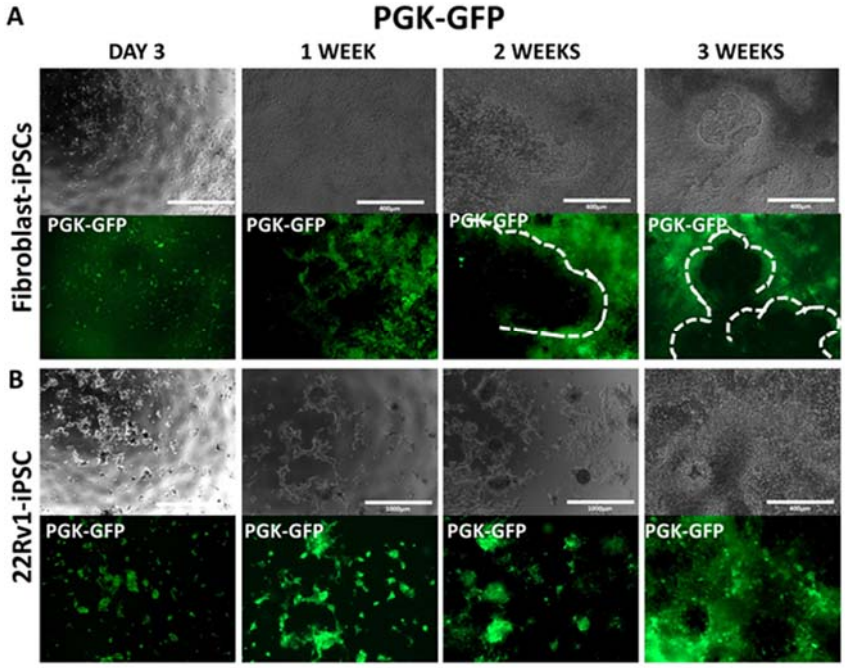


Fig. 6. Control experiment of fibroblasts- and 22Rv1-derived iPSC transduced with housekeeping PGK-GFP reporter during prostate differentiation. Results showed that organoids derived from both cell types similarly express PGK-GFP reporter in a 3-week time-course. However, only fibroblast-iPSC (top) but not 22RV-iPSC (bottom) forms acinar-like structures.

Generation of Caucasian and African-American prostate epithelial cells:

Having established the optimal condition and validate the differentiation of iPSC to prostate epithelial-like cells, we performed prostate differentiation of fibroblast-derived iPSC, two lines each, from Caucasian and African-American individuals (Fig. 7 and 8).

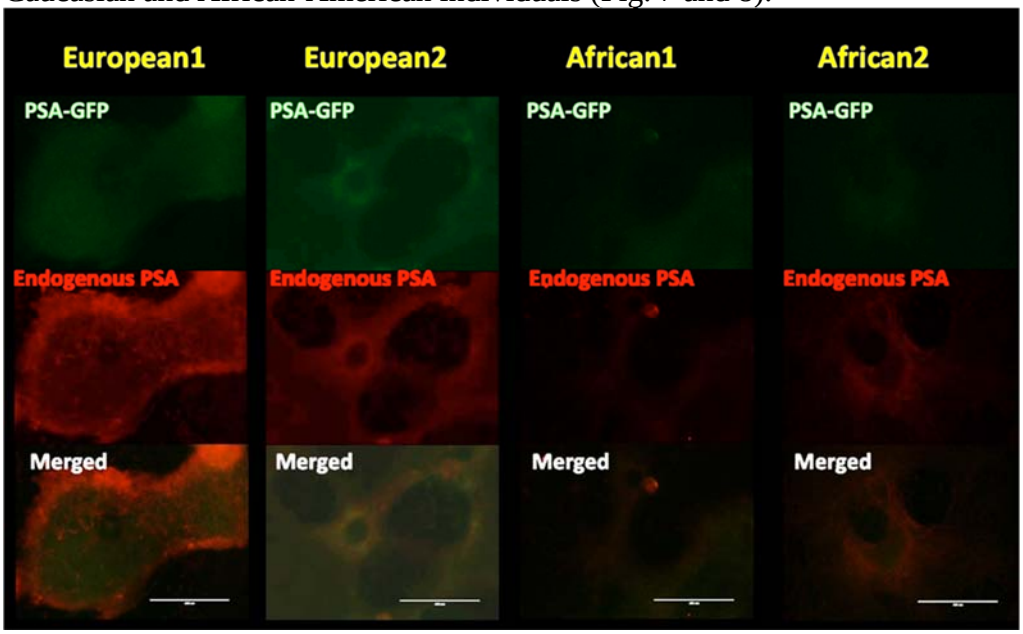


Fig. 7. Prostate differentiation of iPSC clones of two European and two African- selected by low admixtures (Fig. 1) and normal karyotypes. iPSC were transduced with PSA-GFP reporter and subjected to prostate differentiation. Results showed that after 4 weeks, European-derived organoids efficiently formed acinar-like structures and internal ducts. Additionally, European-derived organoid expressed the PSA-GFP reporter and co-localized with endogenous PSA expression. However, African-American derived organoids did not form efficiently acinar-like structures and showed weak expression of both PSA-GFP reporter and endogenous PSC expression.

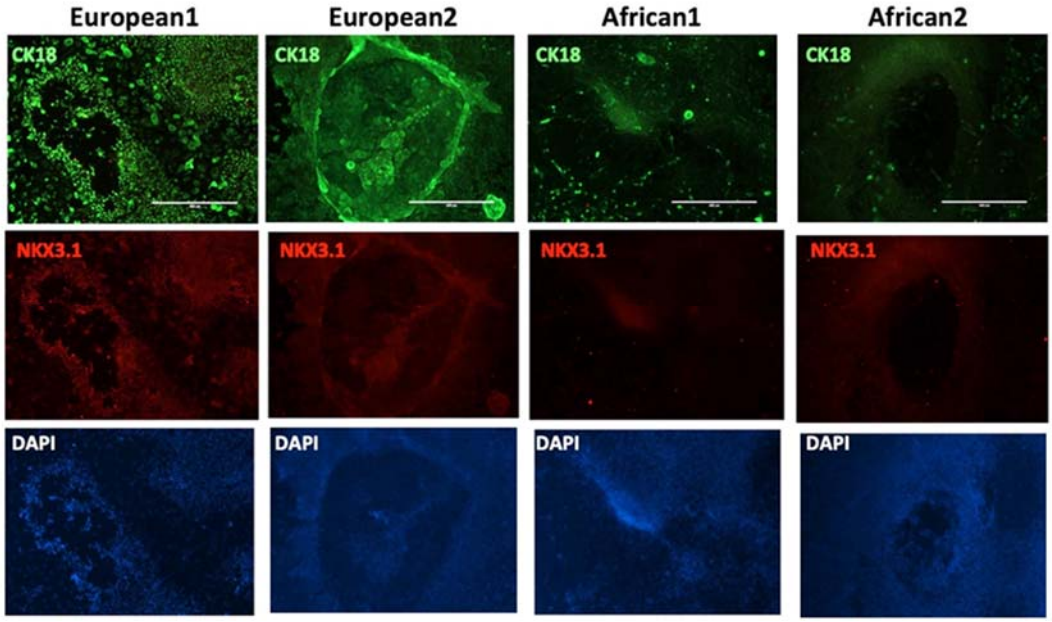


Fig. 8. Immunofluorescence staining of Cytokeratin 18 and NKX3.1 of tw European and two African prostate organoids. European samples show strong expression of both markers and prostate-like morphogenesis. African-American organoids show weak expression and low organization

- **What opportunities for training and professional development has the project provided?**
    - This project has further provided the opportunity for a postdoctoral fellow Dr. Edgardo Castro Perez to acquire skills in iPSC methodology, 2D and 3D organoids differentiation *in vitro* and learn concepts in prostate development and cancer.
- **How were the results disseminated to communities of interest?**
    - A manuscript was in preparation during the last reporting period. However, due to unavailability of prostate media components from Lonza, submitting this manuscript was delayed. With the completion of this phase of the project, we plan to submit the first report in the next 2-3 months.
- **What do you plan to do during the next reporting period to accomplish the goals?**
    - Transcriptome data from iPSC clones and iPSC-derived prostate organoids from European- and African- derived samples.
    - Experiments on potential genetic susceptibility disparities assays of European- and African- derived iPS cells differentiated into prostate organoids.

4. **IMPACT:**
    - **What was the impact on the development of the principal discipline(s) of the project?**
        - A new method for iPSC differentiation into 2D and 3D prostate organoids *in vitro*.
    - **What was the impact on other disciplines?**
        - Nothing to report yet.
    - **What was the impact on technology transfer?**
        - Nothing to Report
    - **What was the impact on society beyond science and technology?**
        - Nothing to Report for this period

5. **CHANGES/PROBLEMS:**
    - **Changes in approach and reasons for change**

      During this reporting period, no changes were made.
    - **Actual or anticipated problems or delays and actions or plans to resolve them**
        - We currently have frozen pellets, DNA and total RNA from the prostate organoids shown in Fig. 7 and 8. The quality of DNA and RNA have not yet been verified. If the quality of DNA and especially RNA is not suitable for sequencing, we expect this to delay the analysis. We plan to propagate the organoids to collect more DNA and RNA.
    - **Changes that had a significant impact on expenditures**
        - The delay in obtaining the media has negative impact on the available funds for the project. I would like to request additional funding, if possible, to complete the final stage of the project, i. e., DNA and RNA sequencing.

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
  - Nothing to Report.
- **Significant changes in use or care of human subjects**
  - None
- **Significant changes in use or care of vertebrate animals.**
  - None
- **Significant changes in use of biohazards and/or select agents**
  - None

6. **PRODUCTS:**

- **Publications, conference papers, and presentations**.
  - **Journal publications.**

    A manuscript is in preparation for publication within 2-3 months:
    Castro-Perez E, Jayanthy A, Setaluri V. **Reprogramming of Induced Pluripotent Stem Cells and *in vitro* Differentiation into Prostate Organoids under Defined Conditions.**
  - **Books or other non-periodical, one-time publications.**
    - 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**
  - Nothing to report.

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

- **What individuals have worked on the project?**

Name:	**Project Role:**	**Researcher Identifier**	**Nearest person month worked:**	**Contribution to Project:**	**Funding Support:**
Vijayasaradhi Setaluri	PI	None	1.2	Overall project administration	This grant
Nihal Ahmad	Co-Investigator	None	0.36	Contributor, supplies PCa cell lines	This grant
Rupa Sridharan	Co-Investigator	None	0.6	Contributor, iPSC characterization: has provided guidance in reprogramming protocols	This grant
Edgardo Castro Perez	Postdoctoral researcher	None	12	Performed most of experiments to date	This grant
Kirthana Prabhakar	Postdegree intern	None	3	Experimental support to Dr. Perez	This grant
Murray Brilliant	PI, Marshfield Clinical Research Foundation subaward	None	0.18	Project oversight: genotyping of fibroblasts	This grant
Lynn Ivacic	Research Associate	None	0.3	Performed genotyping experiments to date	This grant
Terrie Kitchner	Research Coordinator	None	0.12	Coordinated institutional regulatory matters	This grant

- **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?**
  - **Organization Name:** Marshfield Clinic Research Foundation
  - **Location of Organization:** Marshfield, WI

- **Partner's contribution to the projec**t
- **Financial support:** NA
- **In-kind support**: NA
- **Facilities**: NA
- **Collaboration**: Genetic ancestry analyses
- **Personnel exchanges:** NA
- **Other.**

8. **SPECIAL REPORTING REQUIREMENTS** NONE

    - **COLLABORATIVE AWARDS:** *For collaborative awards, independent reports are required from BOTH the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to https://ers.amedd.army.mil for each unique award.*
    - **QUAD CHARTS:** *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*

9. **APPENDICES:** *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*