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<b>14. ABSTRACT</b> 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.					
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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:**

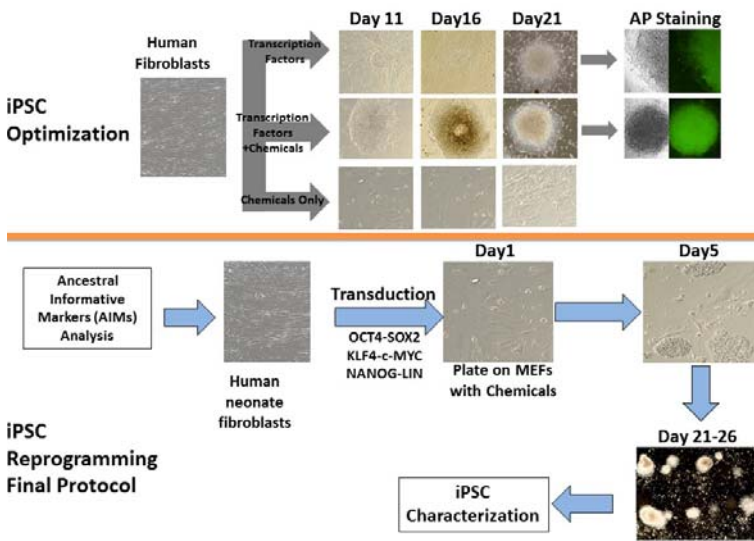
- **What were the major goals of the project?**
  - To establish culture conditions that promote differentiation of human neonatal foreskin skin fibroblast-derived iPSC into cells with characteristics of prostate epithelium.
  - Identify differences in gene expression and epigenetic signatures between 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**

## Task 1.

### A) Establish culture conditions for differentiation of iPSC:

We collected and established 24 primary fibroblast cultures from neonatal foreskin specimens. Using representative Caucasian fibroblasts, first, we optimized the conditions for efficient reprogramming of fibroblasts to induced pluripotent cells (Fig 1, upper panel). We found a combination of classical reprogramming factors plus a chemical cocktail to be most effective in reprogramming. Using this protocol, we reprogrammed 4 fibroblast lines each of African ancestry and European ancestry.

**Figure 1. Upper panel: Optimization of iPSC reprogramming.** First, we decided to investigate which reprogramming conditions were the best for our experiments. We tested if using reprogramming transcription factors (TFs) (OCT4-SOX2, KLF4-c-MYC, NANOG-LIN28) alone or in combination with a 5-chemical cocktail (CHIR, REPSOX, FSK, VPA, TRANYLCIPROMINE) would generate most efficiently iPSC reprogramming from human neonate fibroblasts. We also used chemicals alone as a control. Results showed that colonies with iPSC morphology appear on day 16 when a combination of transcription factors and chemicals were used to reprogram compared to day 21 when TFs alone were used. We also asked if there were differences in quality



of iPSC colonies generated under these two conditions. We used alkaline phosphatase (AP) live staining (green fluorescence) as a marker to evaluate the quality of our iPSCs. We found that iPSC generated with TF and chemicals showed stronger AP staining than colonies generated with TF alone. Furthermore, we observed that iPSC generated with TF and chemicals were more stable and less susceptible to differentiate over several passages (more than 15 passages) than those generated with TF alone. Altogether, these results confirmed that the most efficient and best quality iPSC were generated combining TF and chemicals.

**Lower panel: iPSC reprogramming from skin fibroblasts.** Fibroblasts isolated from human neonatal foreskins were subjected to Ancestral Informative Markers (AIMs) Analysis to verify the genetic background (African or European) and selected for iPSC reprogramming using the optimized protocol and then subjected to alkaline phosphatase (AP) staining, Western blot (WB), stem cell markers with Immunofluorescence (IF) and capacity to differentiate into three germ layers by *in vitro* differentiation assays.

### B) Genetic ancestry determination of fibroblasts:

Genomic DNA isolated from primary fibroblasts was analyzed for 39 ancestry informative markers (AIM). Table 1 shows the details of these markers including genotype, population frequency and estimated ancestry percentage. Table 2 shows the analysis of 24 primary fibroblasts and the samples selected for reprogramming are highlighted.

A	B	C	D	E	F	G	H	I
Sample	Assay	Genotype	Description	Score	Variant	Population Frequency	≥90%	≥80%
Z	rs1006689	C	A.Conservative	0.99499	C/T	AFR 91% C, EAS 81% C, EUR 79% T	AFR	
Z	rs10492585	C	A.Conservative	0.9956	C/T (A/G)	AFR 89% G, AMR 86% A, EAS 91% A, EUR 94% A, SAS 76% A		AFR
Z	rs1198418	A	A.Conservative	0.99683	A/T	AFR 82% A, AMR 72% A, 70% EUR A, EAS 65% T		AFR
Z	rs12609896	AG	B.Moderate	0.91309	A/G	AFR 80% G, EAS 86% A, EUR 81% G, SAS 78% G		X
Z	rs12913832	A	A.Conservative	0.99455	A/G	CEU 95% G, Asian 100% A, African 95% A	X	
Z	rs1409296	A	A.Conservative	0.96479	A/T	AFR 90% A, EAS 97% T	AFR	
Z	rs1426654	G	A.Conservative	0.99465	A/G	CEU 99% A, Asian 95% G, NA 90% A	X	
Z	rs1488994	GT	B.Moderate	0.92005	G/T	AFR 90% G, AMR 76% T, EAS 93% T, EUR 81% T, SAS 74% T	X	X
Z	rs16891982	CG	B.Moderate	0.922	C/G	CEU 95% G, Asian 95% C	X	
Z	rs17138131	T	A.Conservative	0.99576	C/T	AFR 88% T, AMR 92% C, EAS 95% C, EUR 94% C, SAS 86% C		AFR
Z	rs17544484	C	A.Conservative	0.9442	A/C	AFR 96% C, EAS 86% A, EUR 79% C	AFR	
Z	rs1800404	G	A.Conservative	0.9958	A/G (C/T)	AFR 87% C, EUR 79% T		AFR
Z	rs1805007	C	A.Conservative	0.99466	C/G/T	Asian 99% C, NA 99% C, MEX 99% C, African 95% C	X	
Z	rs199138	T	A.Conservative	0.99557	C/T (A/G)	AFR 90% A, AMR 82% G, EAS 95% G, EUR 93% G, SAS 97% G	X	
Z	rs2027760	C	A.Conservative	0.97238	C/T	CEU 99% C, Asian 90% T	X	
Z	rs2120134	C	A.Conservative	0.99464	A/C (G/T)	AFR 88% G, AMR 82% T, EAS 99% T, EUR 91% T, SAS 90% T		AFR
Z	rs213269	C	B.Moderate	0.91438	C/G/T	CEU 100% C, EAS, EUR, and SAS 100% C, AMR 99% C and 1% T, AFR 93% C and 3% G, 4% T	X	
Z	rs2230357	C	A.Conservative	0.94314	C/T (G/A)	CEU 100% C, AFR 98% G and 2% A, all others 100% G	X	
Z	rs2288512	C	A.Conservative	0.97784	C/T	AFR 82% C, AMR 93% T, EAS 80% T, EUR 98% T, SAS 81% T		AFR
Z	rs2424984	CT	A.Conservative	0.97824	C/T	AFR 80% C, AMR 87% T, EAS 79% T, EUR 90% T, SAS 83% T	X	X
Z	rs2426553	G	A.Conservative	0.99645	A/G	AFR 83% G, AMR 93% A, EAS 100% A, EUR 99% A, SAS 98% A		AFR
Z	rs2767564	C	A.Conservative	0.99619	C/T (A/G)	AFR 95% G, AMR 84% A, EAS 100% A, EUR 85% A, SAS 91% A	AFR	
Z	rs2789823	G	B.Moderate	0.90245	A/G	AFR 89% G, AMR 93% A, EAS and SAS 100% A, EUR 99% A		AFR
Z	rs289632	A	A.Conservative	0.99627	A/G	All 100% A except AFR with 14% G and AMR with 1% G	X	
Z	rs3734548	C	A.Conservative	0.99561	C/T	AFR 87% C, AMR 88% T, EAS 84% T, EUR 98% T, SAS 87% T		AFR
Z	rs3747466	C	A.Conservative	0.99756	C/T (G/A)	CEU 100% C, all 100% G except EAS with 7% A	X	
Z	rs3768641	CG	A.Conservative	0.98127	C/G	AFR 96% G, AMR 86% C, EAS 96% C, EUR 91% C, SAS 97% C	X	X
Z	rs3812969	CG	A.Conservative	0.98181	C/G	No pattern observed: AFR 78% G, AMR 89% G, EAS 88% G, EUR 89% G, SAS 83% G	X	X
Z	rs4143483	G	A.Conservative	0.98955	A/G (T/C)	CEU 100% G, all 100% C except ESN 1% T (Esan in Nigeria)	X	
Z	rs4876412	C	A.Conservative	0.99562	C/T	CEU 99% C, AMR 96% C, EUR 98% C, SAS 78% C, AFR 80% C, EAS 78% C	X	
Z	rs4891384	CT	E.User Call		C/T	AFR 89% T, EAS 100% C, SAS 93% C, EUR 96% C, AMR 91% C	?	?
Z	rs6003	AG	B.Moderate	0.89214	A/G	(G;G) unique to Africans except 1% CEU and 5% NA, Asians and NA 95% A, MEX 90% A	X	
Z	rs609096	A	A.Conservative	0.99467	A/G	EAS 96% G, EUR 80% A, AFR 68% A and 32% G		X
Z	rs6137010	C	A.Conservative	0.99564	C/T	CEU and MEX 92% C, 99% of Africans and 100% CEU do not express (T;T), Asians 80% T	X	
Z	rs6586371	G	A.Conservative	0.99609	A/G	AFR 90% G, EAS 85% G, EUR 89% A	AFR	X
Z	rs710080	G	A.Conservative	0.99528	A/G	AFR 75% G, AMR 83% A, EAS 89% A, EUR 98% A, SAS 78% A		
Z	rs7170198	T	A.Conservative	0.99596	C/T	AFR 87% T, EAS 90% C, EUR 90% T	X	AFR
Z	rs7313483	C	E.User Call		C/T	EAS 100% T, SAS 80% T, EUR 77% T, AMR 72% T, AFR 85% C	?	?
Z	rs922452	AG	A.Conservative	0.95311	A/G (C/T)	AFR 95% C, EUR 99% C, SAS 89% C, EAS 91% T	X	X

**Table 1. Representative AIM analysis of a human neonatal fibroblasts.** Each fibroblast sample was subjected to genotyping using AIM analysis of 39 allele specific SNPs (Assay Column) to determine the genetic ancestry. We compared the sample's genotype with the highest allele frequency (≥80-90%) specifically associated with the African populations in the literature. Allele variants associated with Africans (AFR) were quantified for each sample at each marker.

Ancestral African Frequency	90%	80%			
Sample	Alleles	Alleles	Total AFRICAN SNPs/Allele Markers	TOTAL HOMOZYGOS	TOTAL ALLELES
013 (African)	6	9	15	15	30
007 (African)	4	5	9	8	17
008 (African)	4	6	10	8	18
020 (African)	3	6	9	8	17
009 (Caucasian)	3	0	3	2	5
014 (Caucasian)	1	2	3	2	5
024 (Caucasian)	2	1	3	2	5
026 (Caucasian)	3	1	4	1	5
023 (Caucasian)	5	2	7	3	10
004 (Caucasian)	3	2	5	2	7
001 (Caucasian)	4	1	5	3	8
002 (Hispanic)	4	3	7	4	11
003 (Caucasian)	5	0	5	3	8
005 (Asian)	4	1	5	3	8
006 (Caucasian)	3	1	4	4	8
011 (Caucasian)	3	1	4	2	6
021 (Caucasian)	3	1	4	3	7
022 (Caucasian)	3	2	5	3	8
025 (Caucasian)	3	1	4	3	7
027 (Caucasian)	3	2	5	2	7
028 (Caucasian)	5	0	5	2	7
029 (Caucasian)	3	2	5	2	7
030 (Caucasian)	3	2	5	3	8
031 (Caucasian)	4	1	5	3	8

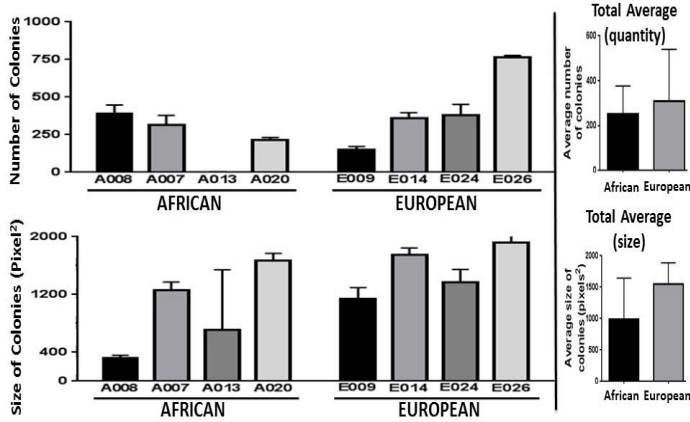
**2. Quantitation of African alleles from 24 human neonate fibroblast samples.** Table shows samples codes with numbers and their self-identified race at the time of tissue collection in parenthesis. African alleles per sample with 80-90% frequency in African-Ancestral populations and total African SNPs/Markers (out of 39 total) markers are shown. Number of homozygous and total number of African specific alleles per sample are

presented. Four (4) fibroblasts with the highest number of African alleles (17-30 alleles) and self-identified as African during the tissue collection were selected for expansion and iPSC reprogramming as African-derived iPSC. Four (4) samples with the lowest number of African-Specific alleles (5 alleles) and self-identified as whites/Caucasian were selected for expansion and iPSC reprogramming as European-derived iPSC.

**C) Differences in reprogramming efficiency between skin fibroblasts of European and African ancestry:**

Four fibroblasts lines each from African and European ancestry individuals were subjected to reprogramming as described in Fig.1 and quantitation of the size and number of iPSC clones is shown in Figs. 4 and 5.

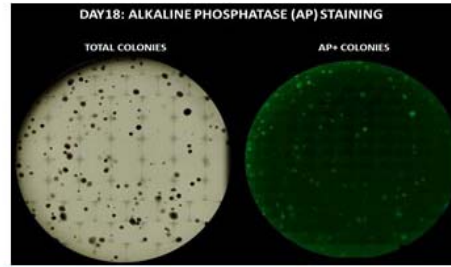
**iPSC Reprogramming Efficiency: African vs European Fibroblasts**



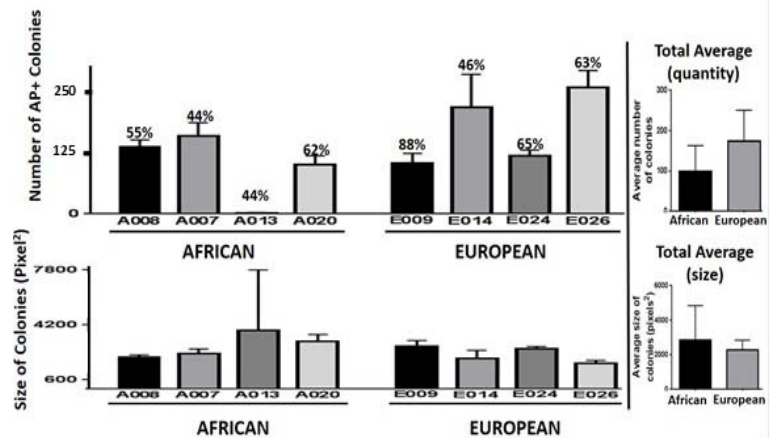
number and size of colonies among the samples but there is no difference associated to Ancestry. However, European-derived iPSC reprogramming efficiency is a slightly higher.

**Figure 5. Alkaline phosphatase (AP) live staining quantification of African and European iPSC colonies.** In previous figure (Figure 4) we showed the quantification of iPSC colonies among African- and European-derived iPSC colonies. Then we questioned if there were differences in AP live staining among the iPSC generated. Plates containing iPSC colonies were AP stained and scanned; and images were processed using ImageJ to quantify the number of AP+ iPSC colonies and their size. We also calculated the % of AP+ iPSC colonies by comparing with total number of colonies. Results showed that 44%-62% of African-derived iPSC, and 46-88% of European-derived iPSC were positive (AP+) for AP staining. In addition, results showed a similar pattern of AP+ iPSC colonies compared with total colonies variations in term of numbers, showing a slightly higher efficiency in European-derived iPSC. However, the size of AP+ iPSC was almost the same for all colonies, regardless of their ancestry.

**Figure 4. iPSC reprogramming efficiency of 4 African and 4 European fibroblasts.** iPSC reprogramming was performed on 6-well plates and we questioned if there were differences in the iPSC reprogramming efficiency within African- and European-derived iPSC. To quantify the efficiency of iPSC reprogramming we counted the number (TOP) and size (BOTTOM) of iPSC colonies 18 days after iPSC induction. 6-well plates with iPSC colonies were scanned at day 18 and images were processed using ImageJ to count the number of colonies and measure their size. Results indicate that there is variation in both

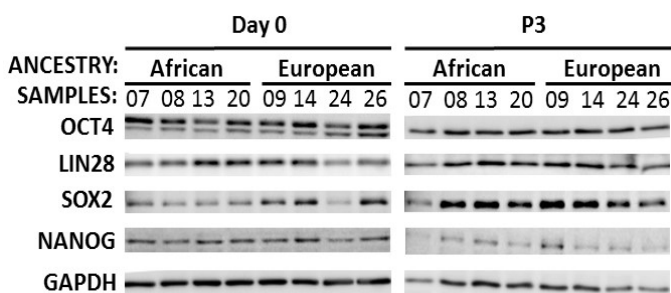


**DAY18, Alkaline Phosphatase (AP) Staining: African vs European**

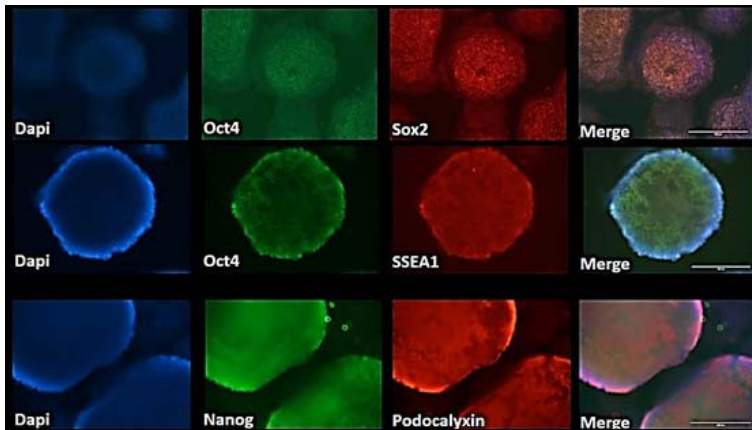


D) Characterization of African and European fibroblast-derived iPSC:

Representative iPSC clones were analyzed by western blotting (Fig. 6) and immunofluorescence staining (Fig. 7)



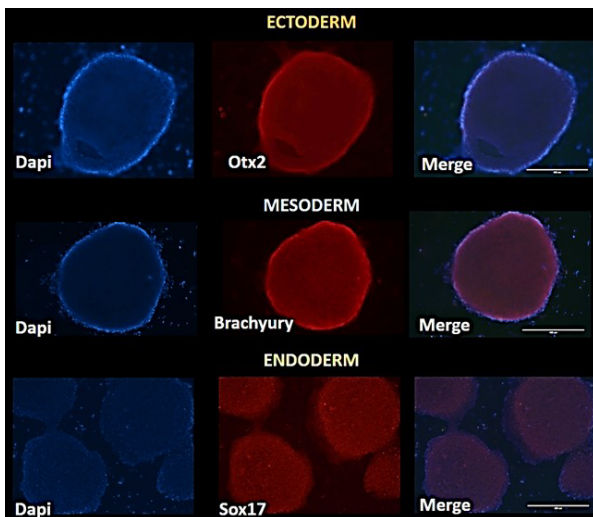
**Figure 6. Expression of reprogramming factors among iPSC by Western blot.** The expression of iPSC reprogramming factors was verified by Western blot. iPSC cells generated from all samples showed stable expression from day 0, corresponding to 5-days transduced fibroblasts and during expansion over passage 3 (p3) of iPSC colonies growing on MEF feeders and stem cell maintenance medium.



**Figure 7. Expression of stem cell specific markers by immunofluorescence (IF).** We have tested multiple iPSC clones to verify that if in addition to reprogramming factors, they also express surface stem cell markers. We determined that all iPSC clones evaluated so far express stem cell surface markers together with reprogramming factors, as shown by IF double labeling.

E) Characterization of iPSC for pluripotency by in vitro differentiation:

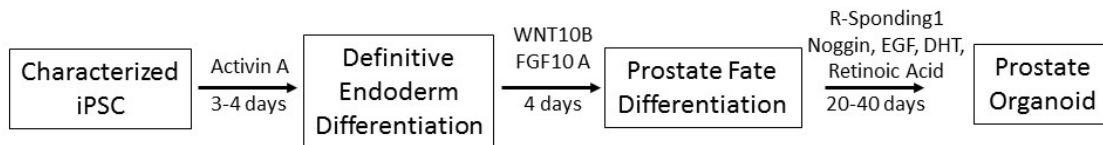
Using a commercially available kit that contains the necessary factors for differentiation of iPSC to cells of the 3 germ layers and antibodies for markers of these lineages, we tested the ability of iPSC we generated to differentiate. Data in Fig. 8 show that the iPSC are able to differentiate in the three germ layers.



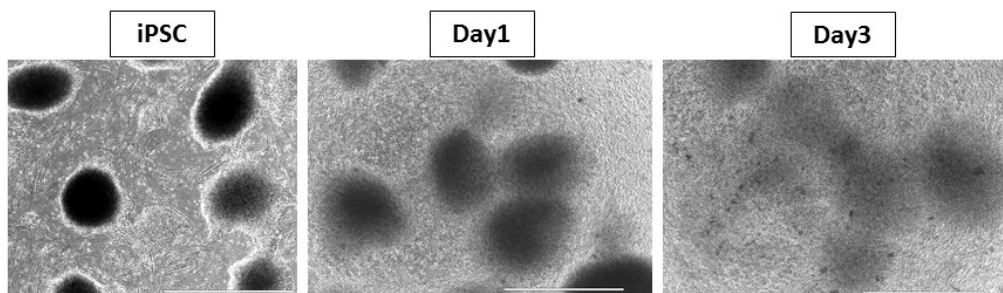
**Figure 8. Characterization of iPSC pluripotency by *in vitro* differentiation assays-followed by immunofluorescence (IF).** We have preliminarily evaluated the differentiation capacity of several iPSC clones generated using *in vitro* differentiation assays (R&D Systems, SC027B) into ectoderm, mesoderm and endoderm. After following differentiation protocols for 3-4 days, iPSC-differentiated colonies were stained to verify that they were expressing their corresponding markers to each specific germ layer: OTX2 for ectoderm, BRACHYURY for mesoderm and SOX17 for endoderm. Results showed that all iPSC colonies so far evaluated have pluripotency to differentiate into three germ layers as

verified by expression of their corresponding makers with IF.

F) Differentiation of iPSC to prostate epithelial cells:



**Definitive Endoderm Differentiation**



We initiated the directed differentiation of iPSC to prostate epithelial cells by a stepwise method by first differentiation into endodermal lineage followed by specific differentiation along prostate



precursor type cells. The preliminary data are shown in Fig. 9.

**Figure 9. Prostate differentiation of iPSC colonies. TOP:** Diagram showing the stepwise prostate differentiation protocol. iPSC colonies are incubated with Activin A in RPMI medium to definitive endoderm differentiation for 3 – 4 days. Ectoderm differentiated cells are driven to prostatic fate by culture in RPMI medium with WNT10B and FGF10 for 4 days. The resultant differentiated cells are cultured in prostate epithelial cell growth medium (PrEGM) and stromal basal medium supplemented with R-Sponding1, Noggin, EGF, 1X B27 supplement, retinoic acid and dihydrotestosterone. **BOTTOM:** Preliminary iPSC differentiation into definitive endoderm over three days. Ongoing experiments will continue the differentiation process to prostate fate and prostate organoids. Each step and checkpoints will be verified by expression of specific markers by immunofluorescence.

- **What opportunities for training and professional development has the project provided?**
  - This project has provided the opportunity for a postdoctoral fellow Dr. Edgardo Castro Perez to acquire skills in iPSC methodology and learn concepts in prostate development and cancer.
- **How were the results disseminated to communities of interest?**
  - Not yet ready for formal dissemination- informally discussed with experts in prostate cancer biology
- **What do you plan to do during the next reporting period to accomplish the goals?**
  - Differentiation of iPSC- tissue culture, qRT-PCR, immunofluorescence and western blotting for markers of prostate differentiation (months 14-20).
  - Isolation of DNA and RNA from 3 iPSC colonies and their differentiated cultures (months 20-24).
  - Prepare and submit RNA and DNA for gene expression profile and CpG methylation analysis (month 24).

#### 4. **IMPACT:**

- **What was the impact on the development of the principal discipline(s) of the project?**
  - Nothing to report yet.
- **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**

- We are comparing directed differentiation of iPSC to prostate epithelial cells using specific signaling molecules with our originally described method using murine urinary genital stromal cells and their conditioned medium
- **Actual or anticipated problems or delays and actions or plans to resolve them**
  - Nothing to Report.
- **Changes that had a significant impact on expenditures**
  - Nothing to Report.
- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
  - Nothing to Report.
- **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.**
    - Although not directly funded by this project, Dr. Edgardo Castro-Perez and I worked on writing a manuscript that describes Dr. Castro-Perez's previous work that is related to this project.  
  
E Castro-Pérez, D E Trejos, T Hrbek, V Setaluri, C W Ramos (2016) Genetic Ancestry of the Panamanian Population: Polymorphic Structure, Chibchan Amerindian Genes; and Biological Perspectives on Diseases. Internet J Biol Anthropol 9: 1-14
  - **Books or other non-periodical, one-time publications.**
    - Nothing to Report.
  - **Other publications, conference papers, and presentations.**
    - Nothing 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?**
  -

<b>Name:</b>	<b>Project Role:</b>	<b>Researcher Identifier</b>	<b>Nearest person month worked:</b>	<b>Contribution to Project:</b>	<b>Funding Support:</b>
Vijayasradhi 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 project**

- **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.*