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TITLE: Differentiation of Neonatal Human-Induced Pluripotent Stem Cells to Prostate Epithelial Cells:
A Model to Study Prostate Cancer Development

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| 14. ABSTRACT We set out to establish conditions for differentiation of human neonatal foreskin skin fibroblast-derived iPSCs into prostate epithelium-like cells and identify differences in gene expression between prostate epithelial cells derived from iPSCs of Caucasian and African-American foreskin fibroblasts. We identified and optimized culture conditions that promote prostate epithelial cell-like differentiation of human iPS clone, IMAR90-4. Our data show that a feeder layer of urogenital mesenchymal (UGSM) cells from neonatal mouse of either gender in combination with neonatal human dermal fibroblasts induced a striking morphological changes that resembles epithelial differentiation with formation of lumen-like structures. We showed requirement of the extracellular matrix of components that promote epithelial-type differentiation. Immunofluorescence and biochemical analyses showed expression of androgen receptor and markers of epithelial differentiation. Analyses of pluripotency marker expression by RT-PCR showed that while human dermal fibroblasts have higher constitutive expression of Nanog, Oct4 and Sox2 compared to IMP90 cells. Our studies showed that black fibroblasts have higher constitutive expression of pluripotency markers than cells from white skin. These data form the basis for studies on the differences in reprogramming of skin fibroblasts and their differentiation into prostate epithelial cells and susceptibility to transformation. | | |
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TABLE OF CONTENTS:

| <i>Item</i> | <i>Page number</i> |
|--|--------------------|
| INTRODUCTION..... | 5 |
| BACKGROUND..... | 5 |
| RESULTS & DISCUSSION..... | 6-7 |
| FIGURES 1-3..... | 7-9 |
| KEY RESEARCH ACCOMPLISHMENTS..... | 10 |
| CONCLUSIONS..... | 10 |
| REFERENCES..... | 10 |
| APPENDICES (N/A)..... | 11 |
| SUPPORTING DATA..... | 11 |

INTRODUCTION:

The incidence of prostate cancer in African-American men is significantly higher with a corresponding higher mortality rate than in Caucasian American males. The reasons for this disparity are not completely understood, but it is likely that inherent genetic (and epigenetic) differences contribute to this difference in prostate cancer risk. Understanding the genetic and epigenetic basis for susceptibility to a cancer that occurs at later decades in life poses a number of technical and conceptual challenges. Recent strategies to study human prostate carcinogenesis by differentiation of human embryonic stem cells (hESC) also have limitations. Induced pluripotent stem cells (iPSCs) offer a useful alternative to hESC. We hypothesized that differentiation of neonatal foreskin fibroblasts-derived iPSCs to prostate epithelial cells is a unique, powerful and innovative strategy for studies on prostate cancer. This approach has many advantages including: 1) unlimited and ongoing source of discarded tissues from both Caucasian and African-American males; 2) obtained temporally closest to the fetal tissue; 3) limited or almost no genetic/epigenetic changes due to environmental exposure; 4) potential to integrate concept in tissue stem cells and cancer stem cells. The specific aims of this application are: 1) to establish conditions that promote differentiation of human neonatal foreskin skin fibroblast-derived iPSCs into cells with characteristics of prostate epithelium *in vitro* and 2) identify differences in gene expression between prostate epithelial cells derived from iPSCs of Caucasian and African-American foreskin fibroblasts.

BODY:

BACKGROUND:

Several *in vivo* and *in vitro* models are currently used to study the prostate and its disorders. These can be broadly categorized into three classes: mouse models, xenograft models and cell culture models. Mouse models such as Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) make use of gene expression or environment based switches to turn on or off key genes within the prostate *in vivo*². Xenograft models make use of a variety of techniques to graft human prostate cells into rodent hosts in order to study development and growth *in vivo*³. Cell culture models use techniques to isolate specific populations of cells from the human prostate or prostate tumors for growth in defined media⁴. These methods, particularly the mouse xenograft models, make use of the fact that mouse mesenchyme has an inductive effect on human cells. Techniques based on this principle have also been used in the study of breast cancer. When cleared mouse breast pads were injected with a mixture of immortalized human fibroblasts and human epithelial organoids, ductal, lobular and acinar structures were formed⁵. These studies indicate the importance of the stroma in the differentiation and maturation of specific cell types and organs.

One of the most important components of the stroma is the fibroblast. Fibroblasts are characterized by their morphology and location in the connective tissue. While they show great diversity in their morphology and gene expression profiles they serve similar functions across various organs. These functions include secreting components of the extracellular matrix (ECM) and basement membrane, a role in wound healing and contraction and regulation of epithelial cell function through the secretion of various growth factors. Fibroblasts have also shown to play a crucial role in both promoting and retarding tumor growth in several cancers⁶. These tumor promoting fibroblasts, also known as carcinoma associated fibroblasts have been shown to play a role in prostate cancer as well. Co-culture studies with prostate carcinoma associated fibroblasts and initiated prostate epithelial cells showed a change in the phenotype of the epithelial cells into that of increased growth, demonstrating the importance of fibroblasts and their particular phenotype within the stroma where they reside⁷.

Previous reports have indicated that rodent urogenital and seminal vesicle mesenchyme is sufficient to induce the transformation of human embryonic stem cells into a prostatic lineage *in vivo*⁸. Based on this, we hypothesized that rodent mesenchyme combined with human dermal fibroblasts could induce the differentiation of induced pluripotent stem cells (iPSCs) into prostate epithelial cells *in vitro*. We also proposed that this model system can be employed to identify differences in gene expression between prostate epithelial

cells derived from iPSCs of Caucasian and African-American foreskin fibroblasts to understand the basis for the higher in prostate cancer risk in African-American men.

RESULTS & DISCUSSION:

Differentiation of human iPSC to prostate luminal epithelial cells:

Dermal fibroblasts were isolated from human neonatal foreskin and cultured in M106 with Low serum growth supplements. Urogenital mesenchyme was isolated from the seminal vesicles and prostate of C57/BL6 neonates between 3 and 5 days old. We used a modified protocol of that previously described⁹. Feeder layers were established from these cells by treating the cells with 10µg/mL mitomycin-c for 3 hours at 37°C and plating each cell type at a density of 2 - 4 X 10⁴ cells/cm² into cell culture treated plastic dishes. iPSCs were obtained from WiCell (Madison, WI) and cultured using a feeder free system consisting of Matrigel and mTeSR1 medium according to conditions described by WiCell. These iPSCs were seeded onto the feeder layers in Prostate Epithelial Growth Medium (PrEGM). Control cultures included dermal fibroblasts and mouse UGSM in PrEGM without iPSCs to rule out the possibility of trans-differentiation of mouse mesenchymal cells to epithelial cells. Between 2-5 days after culture, cells in the iPSC clusters showed flattening and epithelial cell-like morphology with initiation of central lumen-like structure. By day 7, the iPSC clusters flattened out clearing the underlying feeder layer and by day 12, the differentiating iPSC colonies had a well formed lumen-like organization. Such marked morphological transformation of cells were not observed in cultures containing only the feeder layers in PrEGM showing that the structures with lumen-like organization are derived from iPSC, but not the stromal cells of the feeder layer (Fig. 1a).

To investigate the nature of differentiation of the iPSC cells observed, we performed immunostaining of the co-cultures for luminal epithelium marker Cytokeratin18 (K18) and basal epithelium marker Cytokeratin 5 (K5). Staining of the 12 d co-cultures showed that the flattened epithelial structures were positive for the luminal cell marker K18 but not basal cell marker K5 (Fig.1b and c). qRT-PCR analysis showed expression of prostate specific antigen PSA mRNA in total RNA isolated from the iPSC seeded co-cultures but not the feeder layer alone cultured in PrEGM (Fig. 1d).

Requirement of both urogenital mesenchyme and fibroblasts for differentiation of iPSC:

In a series of experiments, we noted that the time for the differentiation of iPSC in the co-cultures varied significantly. We found that with freshly isolated urogenital mesenchyme, the iPSC showed differentiation by day 2. Serial passage of the mesenchymal cells in culture resulted in a delayed inductive effect on the iPSC with a complete loss of their ability to induce differentiation by passage beyond 5. Similarly, a single free-thaw cycle of freshly isolated mesenchyme also resulted in a delay of this inductive effect. We then asked whether urogenital mesenchyme was sufficient to induce differentiation of iPSC by plating them on a feeder layer containing mitomycin-treated UGSM alone or dermal fibroblasts alone or both cell types. As shown in Fig. 2a, no morphological changes similar to those seen with a feeder layer of both cell types demonstrating the requirement of both cell types for the directed differentiation of iPSC.

To test whether providing extracellular matrix will affect the efficiency of differentiation, we plated the iPSC on feeder layer cultured in dishes coated with or without Matrigel. As shown in Fig. 2b, compared to iPSC on a feeder layer in plastic culture dishes, iPSC on feeder layers on untreated glass dishes nor Matrigel-coated tissue culture plastic dishes did not show the distinct morphological differentiation. Although cells on Matrigel showed a tendency to flatten and remain as epithelial cell clusters, no central lumen-like clearing developed.

Next, we asked if conditioned medium from the feeder layers is sufficient to induce differentiation of iPSC on gelatin-coated plates as maintained routinely. Although there was no marked morphological reorganization of iPSC colonies as seen on the feeder layers, culture of iPSC in the conditioned medium for 7 days was sufficient to induce the expression of androgen receptor and prostate specific antigen (PSA), specific markers of prostate differentiation (Fig. 3b). Similarly, growth medium for UGSM also induced epithelial differentiation and expression of androgen receptor, but did not support formation of organized structures (Fig. 3a and c). Biochemical analysis by western blotting (Fig. 3d) showed that the iPSC grown in gelatin with UGSM medium

express AR but also alpha-actinin, a marker of smooth muscle differentiation. These data suggest that secreted growth factors from the co-cultures are sufficient to induce molecular differentiation of iPSC, but cell-cell contact with mesenchymal cells and/or fibroblasts is required for complete morphological differentiation.

Based on these observations, we conclude that iPSCs can be induced to differentiate into prostate luminal epithelial-like cells expressing AR and PSA using a feeder layer formed of human dermal fibroblasts and mouse urogenital mesenchyme. Interestingly, the co-cultures did not require androgens to induce the differentiation of the iPSCs into prostate epithelial cells.

This unique system of differentiation provides an opportunity to exert an exquisite level of control over the prostate epithelial differentiation process. By variously modifying or substituting the components, mechanistic insights into prostate epithelial cell development can be gained.

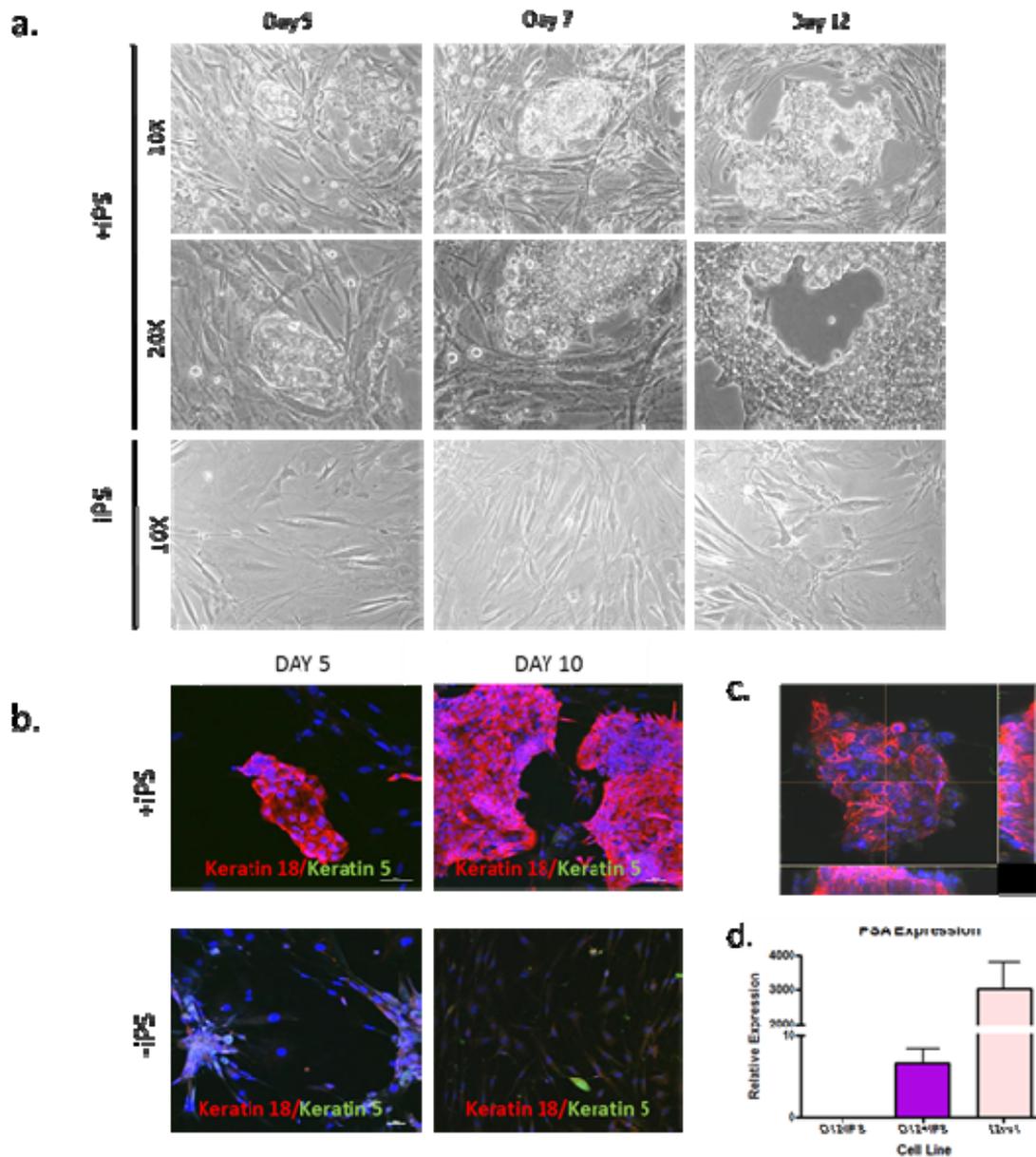


Fig. 1. Differentiation of human iPSC to prostate luminal epithelial cells: a) Morphology of co-cultures containing +/- iPS IMR-90 cells at day 5, 7 and 12 photographed at 10x and 20x magnification. b and c) immunofluorescence analysis of K5 (green) and K18 (red) expression. Nuclei are stained with DAPI. d) qRT-PCR analysis of total RNA from cultures with or without iPSC and prostate cancer cell line 22Rv1 for expression of PSA.

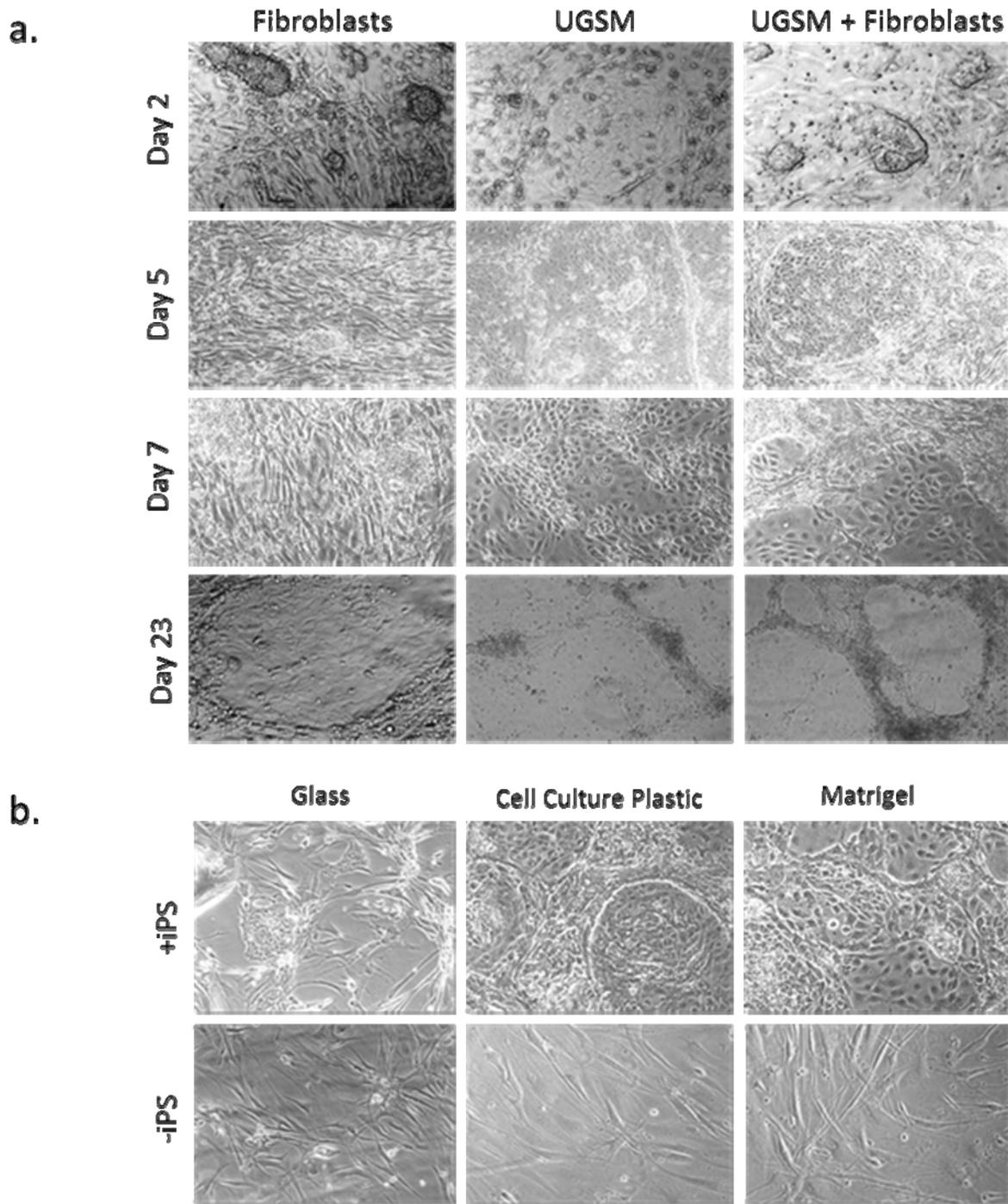


Fig. 2. Prostate luminal epithelium-like differentiation requires both mesenchymal cells and fibroblasts: a) Morphology of co-cultures containing +/- iPS IMR-90 cells at day 2, 5, 7 and 23 on mitomycin-treated fibroblasts or UGSM alone or both cell type. Cultures were photographed at 10x. **b)** Effect of feeder layers grown on uncoated plastic or Matrigel-coated tissue culture plastic substratum on formation of luminal epithelium-like structures from iPSC.

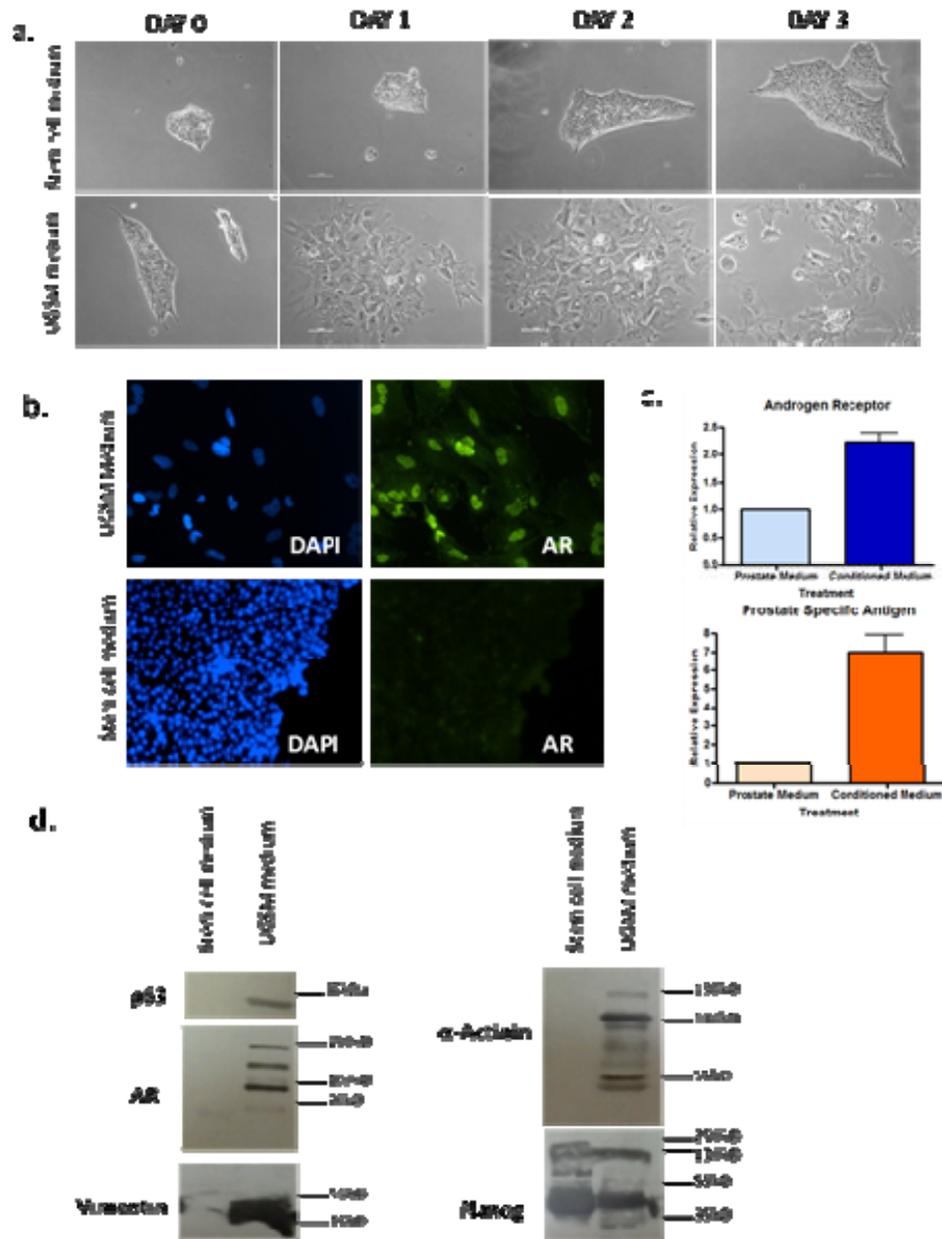


Fig. 3. Effect of conditioned medium from feeder layer on differentiation of iPSC. a) IMR90-4 iPSCs on Matrigel were treated with either UGSM medium (top) or continued in stem cell medium (bottom) at Day 0. Images of the same area were taken every 24 hours for 3 days post-treatment. b) Immunofluorescence analysis of cultures in a for expression of androgen receptor (AR). c) qRT-PCR analyses for expression of AR and PSA in iPSC cultured on gelatin in feeder layer conditioned medium. d) Western blotting analyses for expression of p63, AR, vimentin and alpha-actinin, markers of epithelial and mesenchymal differentiation.

KEY RESEARCH ACCOMPLISHMENTS:

- In this study, we have established optimal conditions for differentiation of human skin fibroblast-derived iPSC to epithelial cells with prostate-like differentiation.
- We have shown that, in an androgen-free medium, iPSC can be differentiated to express androgen-receptor and prostate specific antigen expressing cells using a feeder layer containing mouse urogenital mesenchymal and human dermal fibroblasts.
- Our data show that under optimal culture conditions, human iPSC can give rise to organoid-like structures with luminal differentiation.

REPORTABLE OUTCOMES:

Manuscripts published-

Shekhani, M. T., Jayanthi, A. S., Maddodi, N., and Setaluri, V. (2013) Cancer stem cells and tumor transdifferentiation: implications for novel therapeutic strategies. *American Journal of Stem Cells* 2: 52-61.

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CONCLUSIONS:

The in vitro protocol that we developed to differentiate skin fibroblast-derived iPSC to prostate epithelium with lumen-like structures is a valuable model to investigate the genetic basis of higher risk of prostate cancer among African-American men.

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APPENDICES: None

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