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TITLE: Carcinoma-Associated Fibroblasts from African American Prostate Cancer Promote Aggressive Tumors: Implications for Developing Novel Therapy

PRINCIPAL INVESTIGATOR: Dr. Xiaolin Zi

CONTRACTING ORGANIZATION: The Regents of the University of California, Irvine

Orange, CA

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Xiaolin Zi, Michael Lilly

E-Mail:xzi@uci.edu

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of California, Irvine
101 The City Dr S
Street2: Bldg 55, Rm 260
Orange, CA

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13. SUPPLEMENTARY NOTES

14. ABSTRACT: Metabolic reprogramming is one of the key characteristics of cancer and tumor microenvironment for fueling the rapid and self-sufficient growth of cancer cells. L-3-phosphoserine phosphatase (PSPH) is one of the five rate-limiting enzymes in the biosynthesis of serine from glucose. We are the first to show that PSPH is overexpressed in carcinoma associated fibroblasts (CAFs) and cancer tissues of African American (AA) prostate cancer (PCa) compared to those of European American (EA) PCa and distant carcinoma associated fibroblasts (dCAFs). High PSPH mRNA levels predict poor survival of prostate cancer. Knocking down the expression of PSPH in PCa DU145 cell line exhibits slow growth and overexpression of PSPH in benign associated prostate fibroblasts promote cell growth. In addition, we have developed primary cultures of 119 EA CAFs and matched BAFs and 39 AA CAFs and matched BAFs. Preliminary studies show that AA CAFs can transform tumorigenic growth and promote metastasis of benign prostate epithelial cells BPH-1 by co-inoculation in sub-Renal capsule xenograft experiment. The results suggest that PSPH may be a new target for treatment of PCa and AA CAFs may promote the aggressiveness of PCa.

15. SUBJECT TERMS
PSPH, prostate cancer disparity, TGF-beta

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1. Introduction

Metabolic changes have been recognized as a hallmark of cancer, which include dysregulation of serine metabolism. In general, extracellular serine alone that enters cells via amino acid transporters is sufficient to meet the needs of tumor cell proliferation. However, some tumor cells can increase de novo serine synthesis through glycolysis intermediates even when sufficient extracellular serine is supplied. Actively synthesized serine is utilized for promoting nucleotide synthesis, redox homeostasis, amino acid transport, and folic acid metabolism, thereby enhancing tumor cell proliferation.

Kinseth et al. examined the differences in gene expression between AA and EA PCa by matching for age and pathological stage or Gleason scores as well as tumor-cell content and stroma-cell content. Striking differences in gene expression were observed in the stroma of AA patients relative to EA: 1016 genes with significant differences between the expression of EA and AA patients were observed. The vast majority (82%) of significant differences were down-regulated. In this study, components of extracellular matrix (ECM), mediators of cellular immunity, mediators of the epithelial-to-mesenchymal transition (EMT) and L-3-phosphoserine phosphatase (PSPH) were the top differentially expressed genes in stroma of AA compared to EA men. PSPH was previously described as a gene expression biomarker in tumor tissues for identifying prostate, breast, endometrial and colorectal cancers specifically in AAs. However, our study was the first to show overexpression of PSPH gene in the cancer stroma of AA patients compared to EAs.

Therefore, in this study, we aim to understand the biological functions of PSPH and its regulatory mechanisms in the stroma of AA *versus* EA PCa using primary cultures of CAFs and tissue recombination model in the sub-renal capsule of SCID mice.

2. Keywords

PSPH, prostate cancer disparity, TGF-beta, carcinoma associated fibroblasts

3. Accomplishments

3.1. *PSPH is over-expressed in AA CAFs and prostate cancer cell lines compared to dCAFs and normal prostate epithelial cells*.

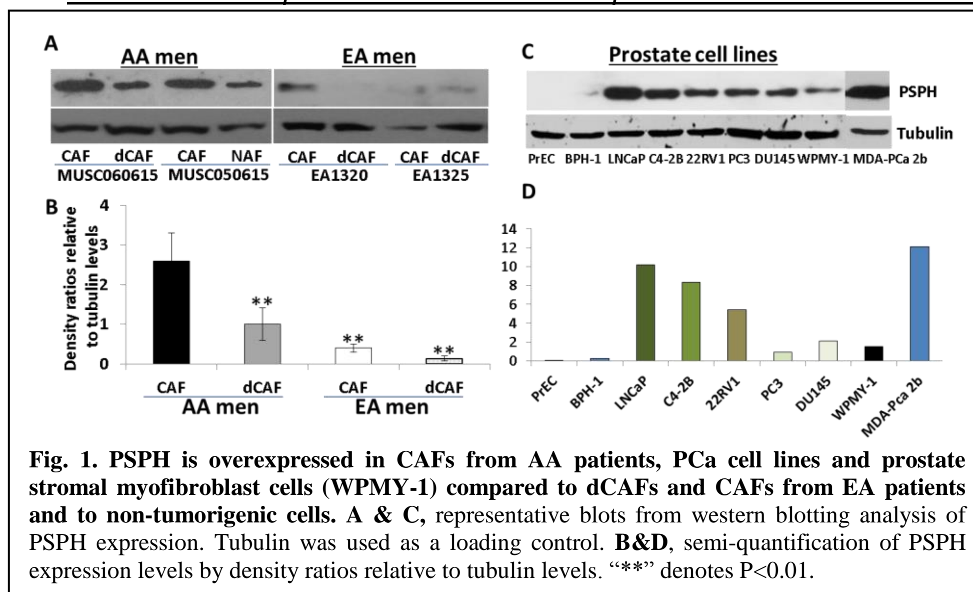

Fig. 1. PSPH is overexpressed in CAFs from AA patients, PCa cell lines and prostate stromal myofibroblast cells (WPMY-1) compared to dCAFs and CAFs from EA patients and to non-tumorigenic cells. A & C, representative blots from western blotting analysis of PSPH expression. Tubulin was used as a loading control. **B&D**, semi-quantification of PSPH expression levels by density ratios relative to tubulin levels. "**" denotes P<0.01.

Figs. 1A & B shows that AA CAFs express higher protein levels of PSPH than EA CAFs and dCAFs. In addition, markedly higher levels of PSPH were observed in PCa cell lines (LNCaP, C4-2B, 22RV1, DU145, PC3, and AA PCa derived-MDA-PCa 2b cells) and WPMY-1 cells, whereas PSPH protein expression was very low or not detected in non-tumorigenic prostate epithelial cells: PrEC and BPH-1 cells (**Figs. 1C & D**).

3.2. *PSPH mRNA levels predict poor survival of PCa patients*. We explored genomic alterations of PSPH in the Cancer Genome Atlas (TCGA) data set. **Fig. 2A** shows that the mRNA levels of PSPH are significantly higher in PCa tissues of AA men than those of EA men. In addition, higher PSPH mRNA levels are associated with poorer survival of PCa patients (**Fig. 2B**). Importantly, PSPH genomic alterations are enriched in fatal metastatic castration resistant prostate cancer (CRPC), including prostate neuroendocrine carcinoma. 17 (22%) of 77 sequenced PCa patients have PSPH gene amplification (**Fig. 2C**). These results suggested that PSPH may contribute to disease aggressive in AA PCa and can serve as a therapeutic target for fatal CRPC.

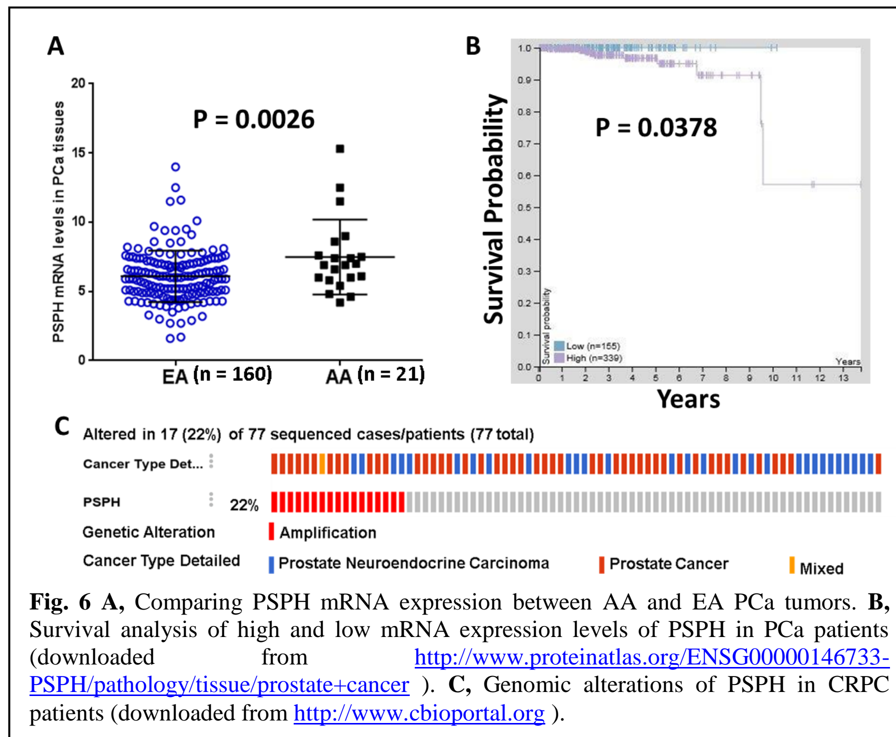

Fig. 6 A, Comparing PSPH mRNA expression between AA and EA PCa tumors. **B,** Survival analysis of high and low mRNA expression levels of PSPH in PCa patients (downloaded from http://www.proteinatlas.org/ENSG00000146733-PSPH/pathology/tissue/prostate+cancer). **C,** Genomic alterations of PSPH in CRPC patients (downloaded from http://www.cbioportal.org).

3.3. *Overexpression of PSPH in dCAFs promotes cell growth and knocking down the expression of PSPH in CRPC cell line DU145 inhibits its growth*. PSH-tert-AR+ cells were derived from normal stromal and immortalized by transfection of tert, which shows low levels of PSPH expression. Fig. 3A shows that PSH-tert-AR+ cells with expression of PSPH grow 3 to 5 fold more faster than PSH-tert-AR+ cells stably transfected with vector control GFP at day 5 (Ps<0.05). **In contract**, knocking-down the expression of PSPH in DU145 cell results in about 18 to 37fold slower growth than DU145 stably transfected with non-silence ShRNA (Ps <0.01). These results suggest that PSPH expression play an important role in cell proliferation or growth.

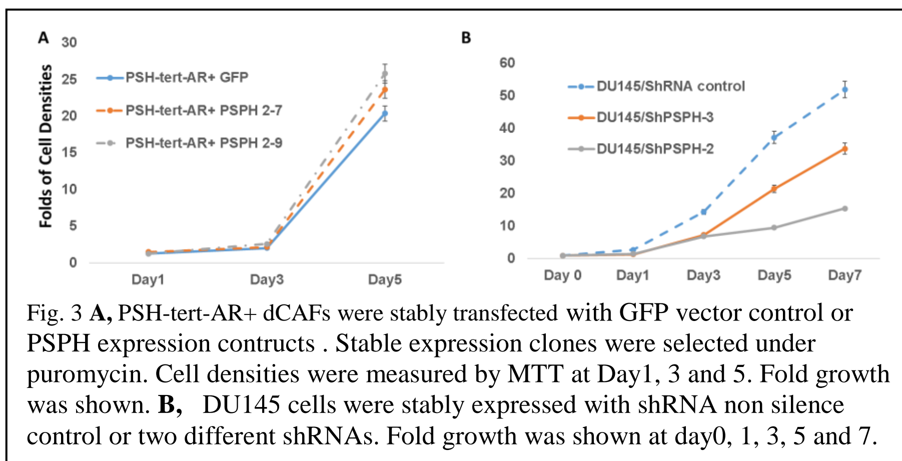

Fig. 3 **A,** PSH-tert-AR+ dCAFs were stably transfected with GFP vector control or PSPH expression contructs . Stable expression clones were selected under puromycin. Cell densities were measured by MTT at Day1, 3 and 5. Fold growth was shown. **B,** DU145 cells were stably expressed with shRNA non silence control or two different shRNAs. Fold growth was shown at day0, 1, 3, 5 and 7.

3.4. *TGF-β inhibits the mRNA expression of PSPH in BPH-1 cells but upregulates PSPH expression in AA CAFs*. **Fig. 4A** Our preliminary studies have shown that 80% (8/10) of AA CAFs secreted higher levels of active TGF β in their conditioned medium (CM) than AA dCAFs, whereas only 20% (1/5) of EA CAFs have higher levels of active TGF β in their CM. Dr. Chung Lee and his associates have shown that TGF-β1 treatment of benign prostate epithelial cells BPH-1 resulted in growth inhibition, whereas aggressive PCa cell lines (PC3, PC3M-LN4, and PC-3M) lost the sensitivity to the growth inhibitory effect of TGF-β1, even though these PCa cell lines secreted more TGF-β than BPH-1cells [92-94]. These results suggested a loss of TGF-β tumor suppressing function during PCa progression. We have found a similar situation of TGF-β's action in myofibroblasts WPMY-1 [95] and AA CAFs in terms of PSPH mRNA expression regulation. TGF-β markedly inhibits the mRNA expression of PSPH in BPH-1 cells (**Fig. 4B**), whereas WPMY-1 cells and AA CAFs are unresponsive to TGF-β mediated suppression of PSPH mRNA expression (**Fig. 4C**). Therefore, it is possible

that myofibroblasts and CAFs may lose their sensitivity to TGF-β inhibition leading to higher expression levels of PSPH and to increased serine/glycine metabolism as observed for AA stroma.

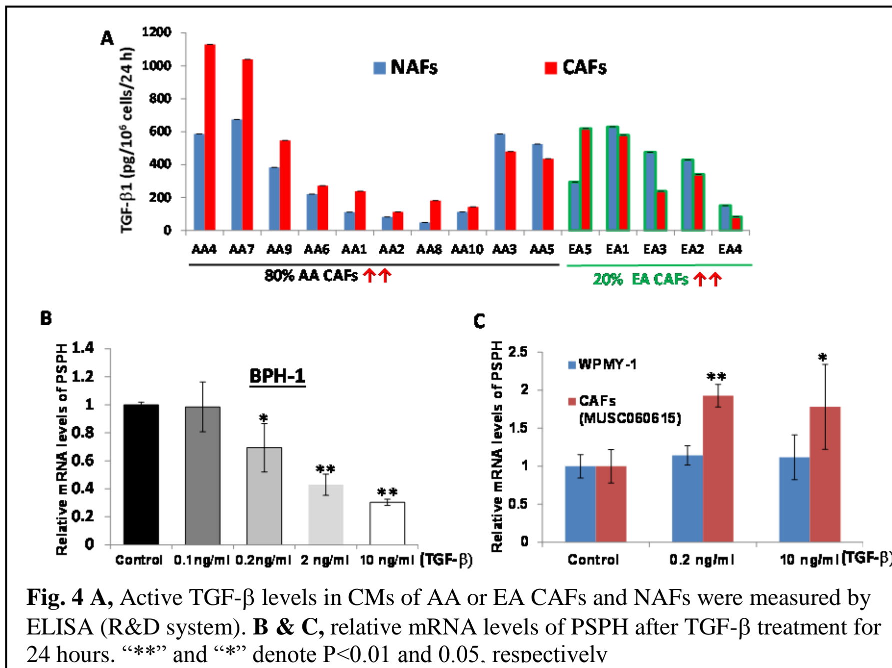

Fig. 4 A, Active TGF-β levels in CMs of AA or EA CAFs and NAFs were measured by ELISA (R&D system). **B & C,** relative mRNA levels of PSPH after TGF-β treatment for 24 hours. "**" and "*" denote P<0.01 and 0.05, respectively

3.5. *The mRNA levels of the integrin α5 (ITGA5) was is down-regulated in AA PCa stroma compared to EA, and inversely correlated with PSPH levels.* To examine molecular mechanisms of PSPH overexpression in the prostate stroma of AA men, we first performed a correlation analysis between differentially expressed genes and PSPH mRNA levels in our microarray dataset [36]. We found that the mRNA levels of PSPH were strikingly inversely correlated with those of ITGA5 with a correlation coefficient of -0.91 in AA stroma (P < 0.001). Compared to EA patients, more AA men expressed higher mRNA levels of PSPH, but low mRNA levels of ITGA5 in the prostate stroma (**Fig. 5**). These results suggested that transcription of PSPH and ITGA5 are inversely co- regulated in AA PCa and that ITGA5 might be associated with suppression of PSPH mRNA expression.

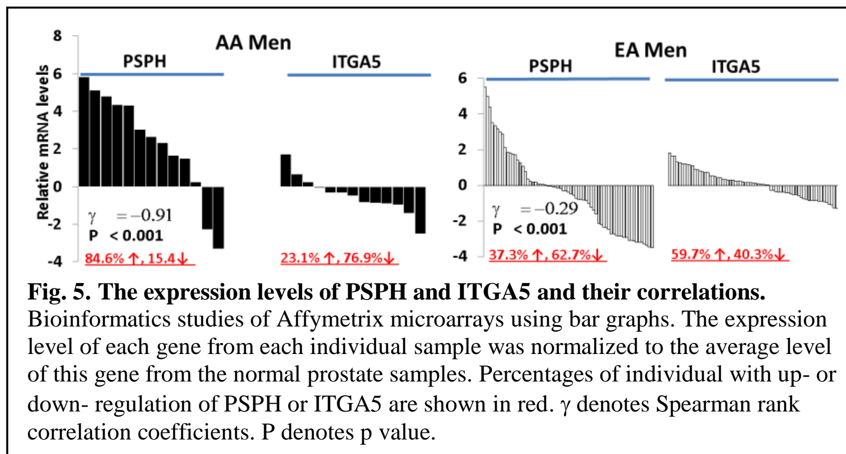

Fig. 5. The expression levels of PSPH and ITGA5 and their correlations. Bioinformatics studies of Affymetrix microarrays using bar graphs. The expression level of each gene from each individual sample was normalized to the average level of this gene from the normal prostate samples. Percentages of individual with up- or down- regulation of PSPH or ITGA5 are shown in red. γ denotes Spearman rank correlation coefficients. P denotes p value.

3.6. *The protein levels of ITGA5 and PSPH are significantly inversely correlated in the prostate stroma of AA patients compared to that of EA patients*. Immunohistochemistry (IHC) staining result with a validated anti-ITGA5 and PSPH antibodies showed that the staining intensity of ITGA5 and PSPH in the stroma of AA specimens is significantly lower and higher, respectively, than those of EA specimens (**Fig. 6**).

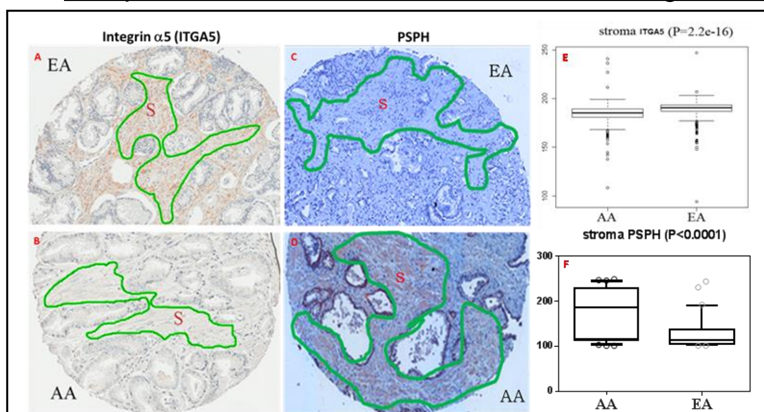

Fig. 6. ITGA5 is down-regulated in AA prostate cancer relative to EA. A to D, representative IHC labelled TMAs from AA patients. S denotes stroma. E&F, Box plots for comparing staining intensities of stroma ITGA5 and PSPH between AA and CA patients (Ps<0.01).

3.7. *ITGA5 suppression up-regulates the mRNA expression of PSPH in NAFs*. We have shown that the mRNA levels of PSPH are highly correlated with multiple integrin genes, including ITGA5. These results strongly support a novel link between integrin expression and the regulation of PSPH mRNA expression. However, the mechanism of PSPH transcriptional regulation remains an unexplored area of research. We therefore have “knocked down” ITGA5 expression in both NAFs and CAFs by siRNA in order to examine whether suppression of ITGA5 expression results in a change in PSPH mRNA expression. **Fig. 7** shows that suppression of ITGA5 expression by siRNA indeed significantly up-regulated the mRNA expression of PSPH in AA NAFs, but not in AA CAFs where PSPH RNA expression is high and the ITGA5 is low. These observations strongly suggest a functional linkage between ITGA5 receptor activity and suppression of PSPH expression consistent with the increased levels of PSPH in CAFs of AA patients where ITGA5 is markedly suppressed. The CAFs of AA patients may, therefore, contribute to aggressive cancer traits through increased production intermediates of DNA synthesis and of high energy TCA intermediates to bring about metabolic reprogramming of tumor cells toward a migratory and metastasizing phenotype.

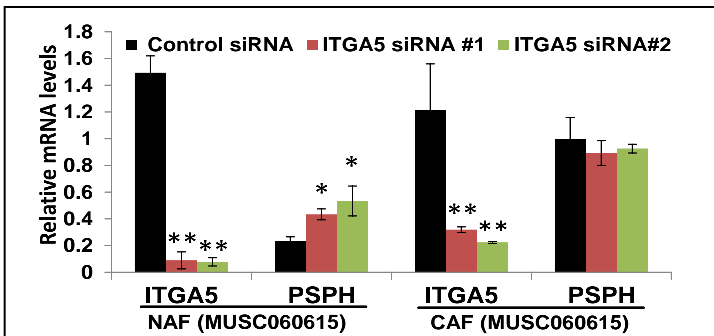

Fig. 5. The regulation of PSPH expression by ITGA5 siRNAs. NAFs and CAFs were transfected by scrambled siRNA control or ITGA5 siRNA. After 24 hours transfection, the relative mRNA expression levels of ITGA5 and PSPH were semi-quantified by real-time RT PCR. “**” and “*” denote P<0.01 and 0.05, respectively

3.8. *Primary cultures of patient-derived CAFs and dCAFs and* ***estimates of ancestry proportions for Native American, European and West African ancestries.*** So far, patient-derived CAFs and matched dCAFs from the contralateral tumor-free portion of the prostate have been derived from **39AAs**: Gleason 3+3 (4), 3+4 (14), 4+3 (10), 4+4 (3), 4+5 (6) and 5+5 (2) and age ranging from 48 to 71 and 119 EAs: Gleason 3+3 (18), 3+4 14), 4+3 (10), 4+4 (3), 4+5 (6) and 5+5 (2) and age ranging from 48 to 71 and **119 EAs**: Gleason 3+3 (18), 3+4 (36), 4+4 (5), 4+3 (38), 4+5 (16) and 5+5 (6) and age ranging from 43 to 73.

By collaboration with **Professor Rick Kittles** at City of Hope and based on the use of a large single-nucleotide polymorphism (SNP) profiles for quantitatively assessing the ethnicity of all AA and EA cells used here, we have obtained estimates of ancestry proportions for Native American, European and West African ancestries in 102 patient samples. **32 patient samples** have more than 68% SNP signatures of **West African ancestries (the majority is 90% or more)**, whereas **64 patient samples** have 58% or more SNP signatures of **European Americans.** Others are Native Americans or half in half with European American.

3.9. *Differential Tumorigenicity observed by in vivo imaging*. Our hypothesis is that the CAFs from prostate tumors of AA patients promote more aggressive disease than those from EA patients. To begin testing this

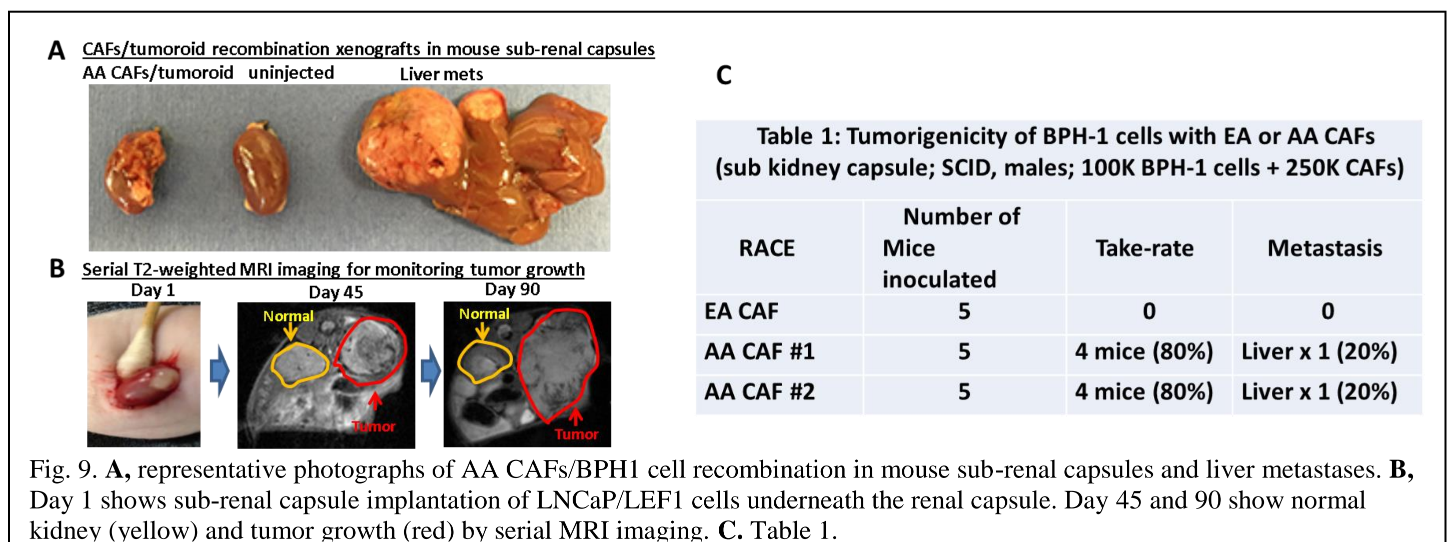

Table 1: Tumorigenicity of BPH-1 cells with EA or AA CAFs (sub kidney capsule; SCID, males; 100K BPH-1 cells + 250K CAFs)			
RACE	Number of Mice inoculated	Take-rate	Metastasis
EA CAF	5	0	0
AA CAF #1	5	4 mice (80%)	Liver x 1 (20%)
AA CAF #2	5	4 mice (80%)	Liver x 1 (20%)

Fig. 9. **A,** representative photographs of AA CAFs/BPH1 cell recombination in mouse sub-renal capsules and liver metastases. **B,** Day 1 shows sub-renal capsule implantation of LNCaP/LEF1 cells underneath the renal capsule. Day 45 and 90 show normal kidney (yellow) and tumor growth (red) by serial MRI imaging. **C.** Table 1.

hypothesis, we compared the tumor initiation and growth of xenografts of nontumorigenic BPH-1 cells using two different lines of patient-derived AA CAFs and one line of EA CAFs. Each of the three lineages was implanted under the kidney capsule of 5 adult male severe combined immunodeficiency (SCID) mice each. The initiation and growth of the xenografts was followed by *in vivo* magnetic resonance imaging (MRI) imaging at the UCI OncoImaging Core using a T9.4 Brunker small animal. As summarized in **Table 1**, 8 of the 10 mice inoculated with AA CAF-BPH-1 combinations led to large xenografts (**Fig. 9**). Furthermore, one inoculation of each AA patient-derived AA CAF line led to large liver metastases (**Fig. 9**) which were confirmed upon necropsy. None of the 5 mice inoculated with EA CAF-BPH-1 combinations developed resolvable tumors and no metastases were found upon necropsy.

4. Impact

4.1. PSPH is overexpressed in prostate cancer and stroma components, which is more significant for AA PCa. PSPH expression contributes to cancer cell growth and predict poor survival of prostate cancer patients. The results suggest that PSPH may be a new target for developing novel therapy in PCa in general and AA populations in particular.

4.2. New mechanisms for regulation of PSPH expression by *ITGA5 and TGF-β may* advance our understanding the aggressiveness of AA PCa.

4.3. We have developed unique resources of CAFs and dCAFs from many different AA and EA PCa patients. These resources provide an important tool for dissecting the stroma regulatory features of PCa of different races.

5. Changes/Problems

The subaward PI Dr. Yang Liu at Chapman University has resigned from her position at Chapman University due to her health issues and family reasons. She also has withdrawn from the collaboration.

One option for this change is to recruit a new co-investigator with extensive experience or expertise on the development of therapeutic nanoparticles.
Dr. Young Jik Kwon, Professor of Pharmaceutical Sciences and Chemical and Biomolecular Engineering at University of California, Irvine has expressed his interest in taking over the work proposed in the Chapman subaward and requires a small increase in the budget.

The other option is to modify specific aim 3 to focus on understanding the regulatory mechanisms of PSPH expression by *ITGA5 and TGF-β* without requiring further budget increasing.

6. Products

6.1. Patient-derived CAFs and dCAFs.
6.2. PSPH expression lentivirus and plasmid constructs

7. Participants & Other Collaborating Organizations

7.1. Medical University of South Carolina
7.2. City of Hope

8. Special Reporting Requirements: N/A

9. Appendices: None