African-American Prostate Normal and Cancer Cells for Health Disparities Research

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Abstract

Prostate cancer is the most frequently diagnosed solid malignancy in men. Epidemiological studies have shown African-American men to be at higher risk for developing prostate cancer and experience higher death as compared to other ethnic groups. Establishment of prostate cancer cell lines paired with normal cells derived from the same patient is a fundamental breakthrough in cell culture technology and provides a resource to improve our understanding of cancer development and pertinent molecular events. Previous studies have demonstrated that conditional reprogramming (CR) allows the establishment and propagation of patient-derived normal and tumor epithelial cell cultures from a variety of tissue types. Here, we report a new AA prostate cell model, paired normal and cancer epithelial cells from the same patient. "Tumor" cell culture AA-103A was derived from malignant prostate tissues, and "normal" cell culture AA-103B was derived from non-malignant prostate tissues from the prostatectomy specimen of an African-American male. These paired cell cultures have been propagated under CRC conditions to permit direct comparison of the molecular and genetic profiles of the normal epithelium and adenocarcinoma cells for comparison of biomarkers, enabling patient-specific pathological analysis, and molecular and cellular characterization. STR confirmed human origin albeit no karyotypic abnormalities in the two cell lines. Further quantitative PCR analyses demonstrated characteristic markers, including the high level of basal cell marker, the keratin 5 (KRT5) in normal cells and of luminal marker, the androgen receptor (AR) as well as the programmed death-ligand 1 (PD-L1) in tumor cells. Although 3-D sphere formation was observed, the AA-103A of tumor cells did not generate tumors in vivo. We report these paired primary epithelial cultures under CRC growth as a potentially useful tool for studies to understand molecular mechanisms underlying health disparities in prostate cancer.

Keywords

American African · Prostate cancer · Primary epithelial culture · Conditional reprogramming · Cell culture

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J. S. Rhim et al. (eds.), *Human Cell Transformation*, Advances in Experimental Medicine and Biology 1164, https://doi.org/10.1007/978-3-030-22254-3_8

Prostate cancer is the most frequently diagnosed solid malignancy in American men with an estimated 164,690 new cases and 29,430 deaths in USA for 2018. According to National Center for Health Statistics, the average annual prostate cancer incidence rate was 208.7 cases per 100,000 black men, 70% higher than the rate in white men. Health disparities studies have shown a higher risk of developing prostate cancer as well as higher cancer-specific death rates in African-American (AA) men as compared to Caucasian-American (CA) men [1]. Although these observations have been attributed to differences in socioeconomic status, such as limitations in access to health care services and delay of cancer diagnosis, environmental exposures and differences in genetics also have been advanced as potential causative factors [1]. AA men were reported to show a higher incidence rate, higher prostate cancer-related mortality rate, and shorter disease-free interval following treatment for localized disease [2–4].

Characteristics of AA Prostate Cancer Cells

Studies performed at centers offering equal access for patients and adjusting for socioeconomic and lifestyle factors have reported differences in survival attributable to tumor biology [5, 6]. In a study of 35 clinical trials, 1843 prostate cancer patients demonstrated survival differences by race [5]. AA men have been reported to demonstrate higher testosterone levels than CA men [6]. Such variations in molecular signaling offer further support for the possibility that genetically based differences in the biology of AA and CA prostate cancers may underlie the observed health disparities.

The normal prostate gland epithelium contains three primary differentiated cell types: luminal, basal, and neuroendocrine cells [7]. The majority of prostate cancers are pathologically classified as adenocarcinoma and display a luminal phenotype. Studies have shown that luminal columnar epithelial cells express secretory proteins (PSA) and other markers (KRT8, KRT18, NKX3.1, and AR). Basal cells localized beneath the luminal layer express markers (KRT5, KRT14, and TP63), but express low levels of androgen receptor (AR), which binds to testosterone and regulates gene expression in normal prostate tissue and prostate cancers. A comparative analysis of malignant and benign prostate tissues from radical prostatectomy specimens has shown higher expression of AR protein in prostate cancer and benign prostate tissues in AA men than in CA men [7]. Such variations in molecular signaling offer further support for the possibility that genetically based differences in the biology of AA and CA prostate cancers may underlie the observed health disparities.

Previous studies have identified differences in gene expression in tumor biopsy specimens from AA men as compared to CA men [7–9]. Microarray analyses of 69 clinically matched prostate cancer patients demonstrated differences in gene expression profiles of prostate tumors from AA and CA men, particularly in genes affecting tumor aggressiveness and metastases [8, 9]. These observations suggest that primary epithelial cultures may offer a useful tool for discovering molecular mechanisms underlying health disparities in prostate cancer.

Establishment of a Pair of Primary Normal and Cancer Epithelial Cell Cultures from AA Prostate Tumors

To investigate a feasibility of establishing paired primary normal and cancer cell lines from prostate specimens, cells were established from radical prostatectomy specimens. The presence of prostatic adenocarcinoma was determined by an experienced pathologist on gross inspection, dissecting tissue separately for the purpose of generating a cell culture as previously described, and the presence of cancer was confirmed by light microscopy [8]. However, conventional cell culture with keratinocyte serum-free medium (K-SFM) is limited by the small number of passages that can be achieved. Expansion of cells derived from prostate tissues that retain lineage commitment and normal growth and differentiation potential has been limited [8].

The Use of Conditional Reprogramming Cellular Technology for Establishment of Paired Cancer and Normal Epithelial Cell Lines from AA Prostate Tumors

The recently developed approach with to indefinitely extend the life span of primary human keratinocytes using both mouse fibroblast feeder cells (J2) and the Rho-associated kinase (ROCK) inhibitor, Y-27632, has been extended to prostate epithelial cells [10–12]. With this conditional reprogramming (CR) technology, we have been able to establish a new AA prostate cell model paired with normal and cancer epithelial cells from the same patient, AA-103A (derived from malignant prostate tissues and referred to as "tumor") and AA-103B (derived from non-malignant prostate tissues and referred to as "normal").

Prostatectomy specimens of the AA patient were annotated with clinical data and de-identified and established as malignant (AA-103A) and normal prostate epithelial cell lines (AA-103B) under an IRB approved protocol. Cells were expanded under CRC conditions as previously reported [11]. Growth characteristics of the prostate epithelial cells are shown in Fig. 1a (2D culture) and b (3D-culture). By day 3 after plating on irradiated feeder cells, epithelial colonies had already formed, expanded, and compressed the adjacent feeder cells. The growth rate and morphological characteristics of the normal and tumor cells were similar.

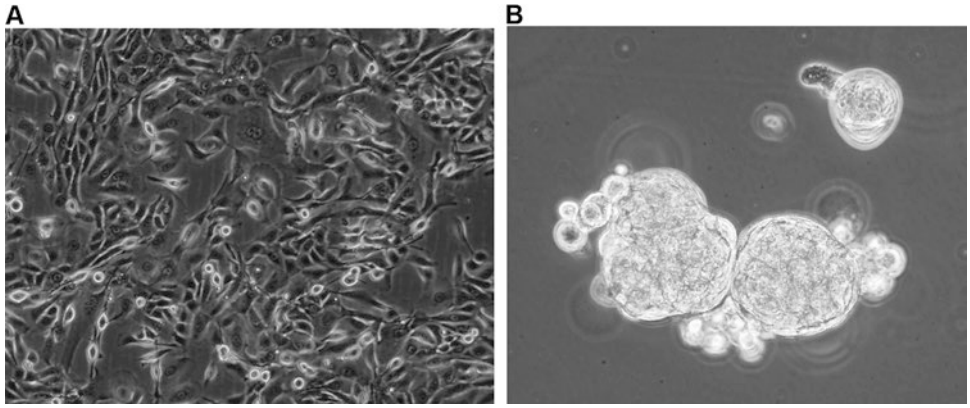

Fig. 1 Characterization of AA-103B (non-malignant—"normal") and AA-103A (cancer) cell lines. The tissues from an African-American patient (44 years old) with prostate cancer were obtained from radical prostatectomy specimens according to Walter Reed Medical Center and Uniformed Services University of the Health Sciences Internal Review Board approved protocols. Fresh prostatectomy tissue specimens were obtained under sterile conditions by an experienced pathologist and used for generating primary cell cultures as previously described [10–12]. Briefly, minced pieces of tissues were distributed to several collagen-coated cell culture dishes with keratinocyte serum-free medium (K-SFM) supplemented with bovine pituitary extract and recombinant epidermal growth factor (Life Technologies, Inc., Gaithersburg, MD). (**a**) The in vitro two-dimensional culture. The prostate epithelial cells were passaged repetitively using trypsinization techniques. The cell numbers were recorded at each passage, and a plot of population doublings versus time (days) was constructed for each cell line. Cells were grown under various conditions: CRC including both feeder cells and Y-27632 in F medium. Only cells grown in F medium containing feeders and Y-27632 continued to proliferate with a constant growth rate. (**b**) Three-dimensional (3-D) cultures. Prostate cells isolated from harvested non-malignant and cancerous tissues, respectively, were plated on a feeder layer of irradiated (4000 rad) Swiss 3 T3 cells (J2 subclone) and grown in low attached plates with F medium containing 10 μmol/L ROCK inhibitor (Y-27632). Small colonies were observed after 2 days. At day 6 there were large islands of epithelial cells that compressed the surrounding feeder cells

Identification of Cell Origin and Cytogenetic Levels

Cells grown in CRC undergo routine mycoplasma testing. To authenticate the novelty of established cell lines, we performed DNA fingerprinting. STR fingerprinting analysis, verifying that these two prostate cell cultures were derived from the same patient and were unique (Fig. 2). The prostate cell cultures have 15 identical *STR* loci and the Y-specific Amelogenin locus, thereby verifying genetic identity. DNA fingerprinting analysis at nine STR loci and at the Y-specific Amelogenin locus (Cell ID System; Promega) also showed that immortalized CRC cultures were not contaminated with another cell line during prolonged passaging. Karyotype analysis was also performed at early and late passages. The data revealed that both normal and tumor cells retained a diploid karyotype. The results for prostate cells are shown in Fig. 3 and verify that their chromosomes are structurally and numerically normal, with a 46, XY karyotype.

Molecular Characterization of AA Cell Lines

Previous studies have shown that the growth rates of the normal and tumor cells were similar; however, normal and tumor cells differed in the expression of several markers [12]. Analysis of cells taken from CRC conditions revealed that tumor cells differentiated into a luminal phenotype. To characterize both prostate normal and cancer epithelial cells, using RT-PCR we examined the expression level of marker genes associated with growth, migration, invasion, and metastases, including PD-L1, PAI1, htert, p63, KRT5, TIMP3, and AR (Figs. 4 and 5). The data showed that normal cells expressed high levels of basal markers TP63 and KRT5, but very low levels of luminal marker AR (Fig. 5). These data suggest that both phenotypes, basal and luminal, are present in CR cultures. AR, PAI1, TIM3, and PD-L1 were upregulated and p63 and KRT5 were downregulated in AA prostate cancer cells (103A). Htert

103A (p8)

DNA Analysis

	D3S1358	D7S820	vWA	FGA	D8S1179	D21S11	D18S51	D5S818	D13S317	D16S539
SMP1	15, 16	10, 12	16	22	15	29, 32.2	15, 17	9, 13	11, 13	8, 9
REF	NA*	NA	NA	NA	NA	NA	NA	NA	NA	NA

DNA Analysis

	TH01	TPOX	CSF1PO	AMEL	Penta D	Penta E
SMP1	7, 9.3	8, 11	11	X, Y	7, 8	5, 11
REF	NA	NA	NA	NA	NA	NA

103B (p8)

DNA Analysis

	D3S1358	D7S820	vWA	FGA	D8S1179	D21S11	D18S51	D5S818	D13S317	D16S539
SMP1	15, 16	10, 12	16	22	15	29, 32.2	15, 17	9, 13	11, 13	8, 9
REF	NA*	NA	NA	NA	NA	NA	NA	NA	NA	NA

DNA Analysis

	TH01	TPOX	CSF1PO	AMEL	Penta D	Penta E
SMP1	7, 9.3	8, 11	11	X, Y	7, 8	5, 11
REF	NA	NA	NA	NA	NA	NA

Fig. 2 Short tandem repeat (STR) analysis. Both the AA-103A and AA-103B cultures were examined for STR patterns. DNA fingerprinting of early passage (p8) prostate cells showed nine identical *STR* loci and the Y-specific Amelogenin locus, thereby verifying their genetic identity. Data are presented as mean SEM. Briefly, the analysis was performed using a commercially available kit (Cell ID System; Promega Corporation, Madison, WI), and the data were matched with cell lines in the ATCC database. The *STR* markers include *CSF1PO*, *TPOX*, *TH01*, *vWA*, *D21S11*, *D16S539*, *D7S820*, *D13S317*, and *D5S818, in addition to* the Amelogenin locus. The PCR amplification was performed according to the manufacturer's recommended protocol. Detection of the amplified fragments was achieved with the ABI 3100 genetic analyzer (Applied Biosystems). Data analysis and allele size determination were performed using GeneMapper Software (Applied Biosystems)

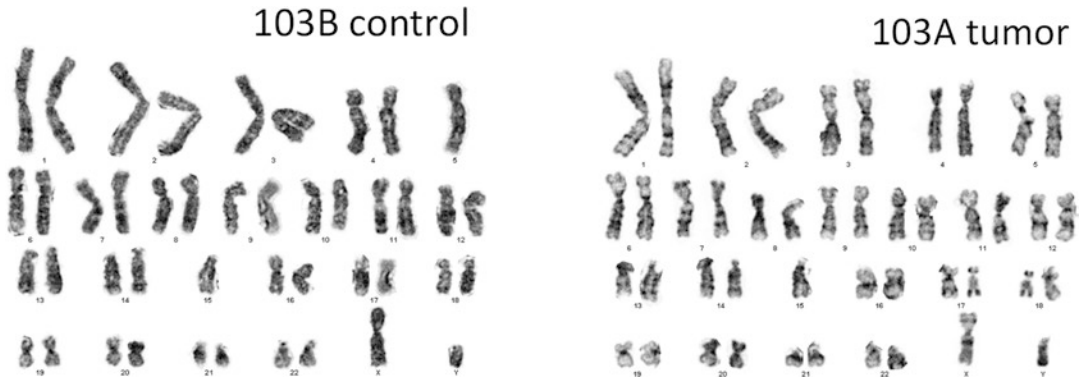

Fig. 3 Cytogenetic analysis. Chromosome counts, ploidy distribution, and Giemsa (G)-banded karyotypes were prepared by standard protocol as described previously [12]. Chromosomal analysis of normal prostate cells AA-103B revealed a normal 46, XY karyotype

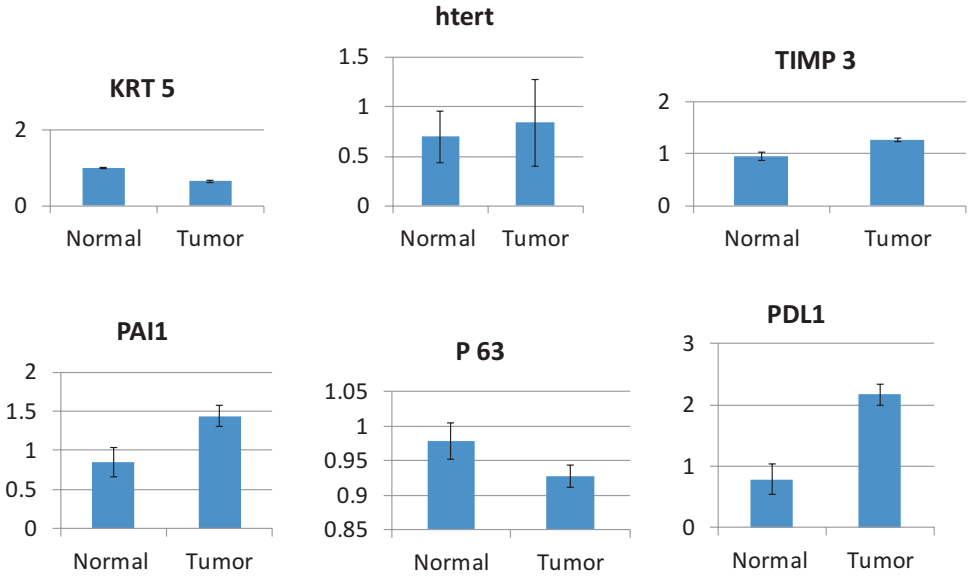

Fig. 4 Quantitative RT-PCR. Total RNA extracted from cells was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time PCR (qRTPCR) was performed in triplicate using TaqMan Gene Expression Assays (Applied Biosystems) on the Applied Biosystems 7900HT Fast Real-time PCR System using standard mode. Genes include AR, PAI1, KRT5, TIM3, p63, PD-L1, and Hert. GAPDH was used as an endogenous control to standardize the amount of sample added to the reaction for relative values of the amount of target cDNA

activity was similar in both cell lines. Interestingly, PAI1 and PD-L1 levels are significantly higher in tumor cells compared to normal. Furthermore, western analysis of both CR lines expressed high levels of PSA but showed a low level of AR in normal cells and higher level in tumor cells comparable to the established cancer cell line LNCaP (Fig. 5). These observations show that primary epithelial cultures may offer useful markers for discovering molecular mechanisms underlying health disparities in prostate cancer.

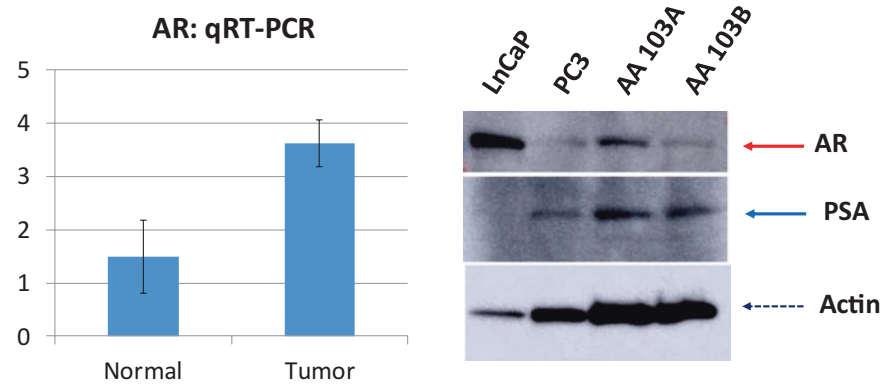

Fig. 5 The expression levels of AR gene (RT-qPCR) and protein (Western blotting). RT-qPCR was performed as described in Fig. 4. PSA and AR protein expression levels were determined by Western analysis. Actin was used as a loading control

Examination of Tumorigenicity In Vivo

To examine capacity of cells to form xenograft tumors, the prostate cancer cells were cultured in conditional medium on low attachment dishes. Within a few days, irregular spheres were formed in conditioned medium on low attachment dishes (Fig. 6a, b). Cells were then injected s.c. into nude mice. After 2 weeks, two of the five mice develop tumors at injected sites measuring 30 mm^3 and 50 mm^3. The tumors were small and after another week started to regress in size. We attributed this effect to immune responses to these cells at the injected sites in nude mice and sacrificed the mice to harvest tumors. Small tumors were obtained, and histopathologic examination did not confirm evidence of malignant growth (Fig. 6c). The experiment was repeated using severe combined immune-deficient (SCID) mice, but did not see tumor growth. We are aware that the yield of tumorigenic cancer-derived cells using CRC is in the 40% range overall.

Discussion and Conclusion

The most aggressive cancers may present as locally advanced disease precluding prostatectomy for their standard of care. However, the lack of availability of primary epithelial cultures has decelerated our understanding of molecular mechanisms underlying prostate cancer progression. Therefore, establishment of cell lines from patients with more aggressive disease is imperative.

Growing primary cultures of human prostatic epithelial cells from prostatectomy specimens is a major challenge due to the complex heterogeneity of primary tumors, a low success rate (1–10%) and technical hurdles. The slow growth rate of non-malignant epithelial cells may bias the selection of cells toward more rapidly growing phenotypes. Recently, an innovative technology, CRC, has been developed for growing primary human keratinocytes from patient's specimen using both mouse fibroblast feeder cells (J2) and the Rho-associated kinase (ROCK) inhibitor [10–12]. Appling a CRC approach, we were able to establish a new AA prostate cell model paired with normal and cancer epithelial cells from the same patient. The direct comparison of the molecular and genetic profile of the normal epithelium and adenocarcinoma cells verified their genetic identity as a human origin. Karyotype analyses revealed that both normal and tumor cells retained a diploid karyotype. Molecular characterization demonstrated that normal CR cells expressed high levels of basal cell markers, including KRT5 and TP63, but low AR. Tumor CR cells expressed a significantly high level of luminal marker, AR, as well as immune checkpoint and EMT markers (PD-L1,

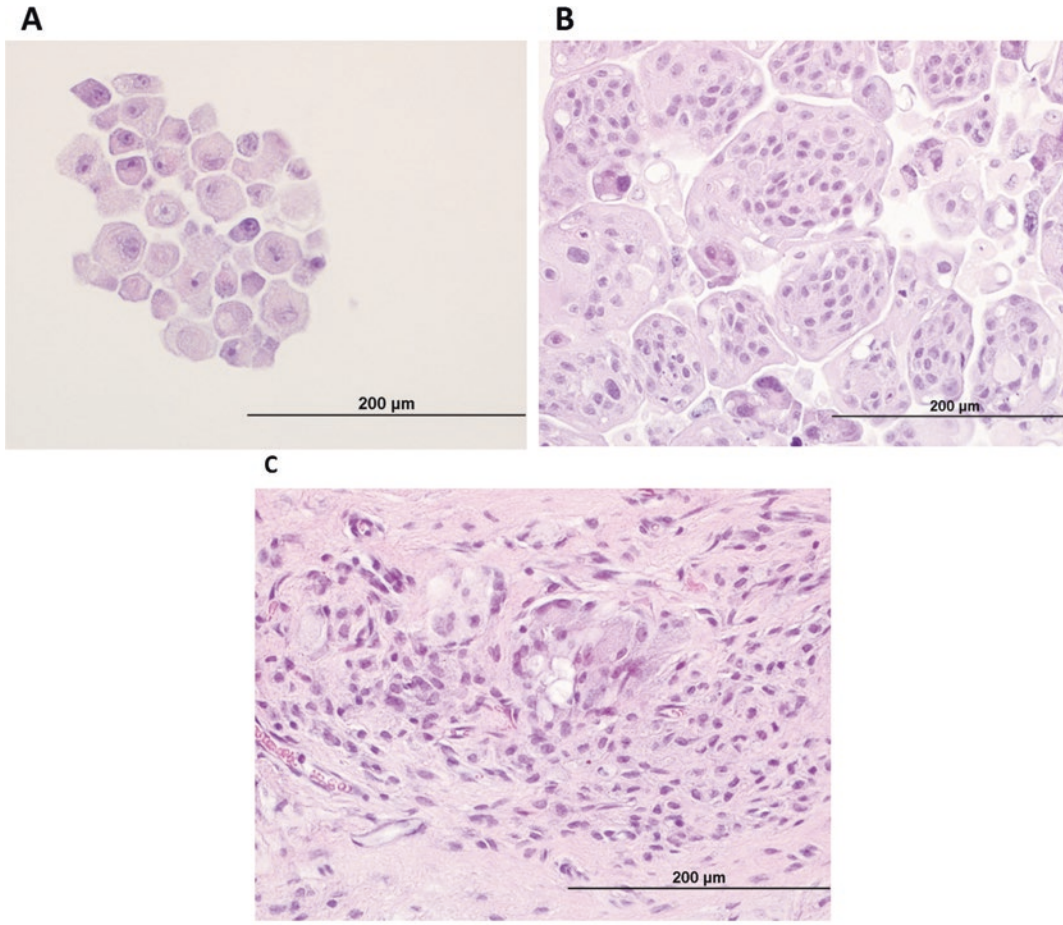

Fig. 6 Tumorigenicity in SCID mice. (**a**) Prostate CRCs in 2D culture in FY medium, 40X; these cells were used to make spheres. (**b**) Prostate CRCs in sphere condition, 40X. The spheres were cultured in conditioned medium without Y-compound on low attachment dishes. The spheres were injected s.c. into nude mice. After 2 months, two of the five mice develop tumors at injected sites measuring 30 mm^3 and 50 mm^3. (**c**) Histopathologic examination of tumors. The spheres were injected in nude mice. The prostate cancer cells made irregular spheres. Small tumors were subjected to histopathologic examination but did not confirm evidence of malignant growth

TIMP3, and PAI1), while basal cell markers are dramatically decreased. However, tumors were not generated in vivo albeit luminal markers were expressed, consistent with findings that the yield of tumorigenic cancer-derived cells is in the 40% range overall.

Tissues derived from AA patients provided the initial reagents for proof-of-principle studies establishing and characterizing paired AA cell lines. The cells may be useful for enhancing research capabilities of basic scientists and/or provide novel tools to pharmaceutical companies for preclinical oncology studies and drug development relevant to health disparities. Overall, the studies performed with cells provide proof of principal for developing additional prostate cell lines for research to facilitate the reduction of cancer health disparities.

Acknowledgments This work was supported by the NIH/NCI SBIR contract number HHSN261201600038C to Shuttle Pharmaceuticals, Inc.

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