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TITLE: Adipose Stem Cell-Based Therapeutic Targeting of Residual Androgens in African Americans With Bone-Metastatic Prostate Cancer

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14. ABSTRACT The disproportionate incidence and mortality of prostate cancer (CaP) among African Americans (AA) in comparison to Caucasian American (CA) are not well understood. It is believed that high circulating androgens reported in AA men may account for such racial disparities. It has been shown that metastatic tumors maintain functional androgen receptor signaling, suggesting that local (<i>intracrine</i>) androgens may contribute to the outgrowth of 'castration-adapted' tumors under androgen deprivation therapy (ADT). Evidence exists for direct correlation between increased obesity and body-mass-index (BMI), which is significantly higher in AA-men, and the risk for aggressive CaP. Active steroidogenic pathways are active in adipocytes and adipose-derived mesenchymal stem cells (ASCs) are often recruited to tumor-stroma. <i>Our goal will be to exploit the tumor-tropism of normal ASCs to deliver androgen inactivating genes to tumor microenvironments and enable an effective treatment strategy against CRPC.</i> This will be achieved by: (a) investigate if "intracrine" production of testosterone by osteotropic ASC ^{AA} modulates growth and metastatic potential of CaP cells under ADT in vitro and in vivo; (b) determine if α -HSD-expressing osteotropic ASC ^{Cont} will nullify the ADMSCAA-mediated CaP cell growth and metastasis in vitro; and (c) examine the efficacy of therapeutically engineered ASC ^{Cont} to target and inhibit CaP tumor growth under CRPC <i>in vivo</i> . The proposed work will be <i>innovative</i> , because it capitalizes on an adjuvant approach for ADT by tumor-site specific inactivation of androgens. Considering the aggressive nature CaP, the outcome of our study is expected to have a positive impact on establishing preventive and/or therapeutic intervention strategies to reduce or circumvent PC, especially among AA-men.					
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Introduction:

Race, a Risk Factor for Prostate Cancer (CaP). In the US, African Americans have the highest annual incidence of CaP, at 272 new cases per 100,000 men [1]. In 2009, 230,000 men were diagnosed with CaP, and more than 38,000 afflicted men are expected to die [2]. Although the etiology remains largely unknown, racial make-up has been identified as one of several risk factors for CaP. African-American (AA) men bear a disproportionately heavy burden from this disease with incidence and mortality rates over 50% higher than Caucasian American (CA) men [3, 4]. Furthermore, AA men are more likely to develop CaP at an earlier age, have higher rate of Gleason-7, aggressive tumors, and metastasis, and exhibit a poorer survival rate than CA males [3-7]. Socioeconomic and environmental factors, such as diet, access to care, and screening, have been cited as factors contributing to the more clinically aggressive CaP in AA patients [8, 9]. Family history accounts for 5-10% of total CaP cases [8, 9], and it does not differ among AA, Asian Americans, and CA men [10, 11]. A more biologically aggressive CaP has been proposed as one possible explanation for the younger age at presentation and disease progression in AA men compared with CA men [12, 13].

Obesity and BMI as Risk Factors for CaP progression in AA men. Prostate cancer incidence and mortality rates correlate well with the average intake of fats, including polyunsaturated fats [14]. *In vivo* and *in vitro* models have demonstrated a decreased rate of proliferation of prostate tumors with reduced fat intake [15-17]. One meta-analysis showed a 5% excess risk of developing prostate cancer for each 5 kg/m² increment of BMI [18]. When disease stage was considered, the analysis showed a rate ratio for advanced cancer of 1.12 per 5 kg/m² increment. An analysis from the CaP Prevention Trial noted that compared with men with a BMI below 25 kg/m², those with a BMI above 30 kg/m² had an 18% decrease in the risk of low-grade cancer, but a 29% increase in the risk of high-grade cancer [19]. In addition, obesity has been linked to aggressive CaP [20] and increased BMI has also shown a positive correlation to Gleason score and positive surgical margins [21]. The latter is critical as it can be an indicator for disease relapse. However, the mechanisms linking obesity to CaP development and progression are not fully understood. Since the prevalence of obesity is significantly high in AA men [22], accounting for 37.3%, unraveling such mechanisms is of paramount significance.

“Intracrine androgens” and CaP progression. Androgen-deprivation therapy (ADT) has been the mainstay treatment for patients with metastatic CaP [23]. Although initially effective, hormonal therapy is marked by progression to castration-resistant prostate cancer (CRPC) over a period of 18–20 months, with median survival of 1–2 years. Importantly, large body of evidence indicate that in the setting of ‘castrate’ serum testosterone levels, prostatic androgen concentrations remain at approximately 10–25% of the levels found in untreated patients [24–26] well within the range capable of mediating continued androgen-receptor (AR) signaling and gene expression [27]. Moreover, residual intra-prostatic androgens are implicated in nearly every mechanism whereby AR-mediated signaling leads to the development of castration-resistant disease [28]. The increased expression of androgen-metabolizing genes within castration-resistant metastatic tumors [29] strongly suggests that up-regulated activity of endogenous steroidogenic pathways is driving the outgrowth of ‘castration-adapted’ tumors. The source of residual androgens within the prostate tumors of castrate men has not been fully elucidated, but has been attributed to the uptake and conversion of circulating adrenal androgens [30]. Whether the de novo biosynthesis of androgens from cholesterol or earlier precursors occurs within castration-resistant metastases is not known [28] but has significant implications for treatment strategies targeting sources of androgens exogenous to the prostate versus ‘intracrine’ sources active within the actual metastatic tumor microenvironments.

Body

Specific Aim-2:- Determine if α -HSD-expressing osteotropic ADMSC^{Cont} will nullify the ADMSC^{AA}-mediated CaP cell growth *in vitro*. Based on our preliminary studies, the working hypothesis here is that only a selective subset of ADMSC^{Cont} has higher propensity to adhere and transmigrate through human bone marrow endothelial cells (BMEC) towards CaP cells *in vitro*. Osteotropic ADMSC^{Cont} will be genetically engineered to express α -hydroxysteroid dehydrogenase (α -HSD), a testosterone and DHT inactivating enzyme, and examined for their ability to suppress ADMSC^{AA}-mediated CaP cell growth *in vitro*.

Task-4: Carry out *in vitro* assays to select for those ADMSC^{Cont} cell subpopulations, which possess increased osteotropism and tumor-homing potential (ADMSC^{Sel} cells)-months 9-14

4.1. **Transmigration experiments in trans-well culture (TWC) chambers containing prostate cancer, with fluorescent-labeled ADMSC^{Cont} on top and CaP CM in bottom.**

4.2. **Harvest the transmigratory ADMSC subpopulations by FACS sorting and grow to large scale to obtain several ADMSC clones that possess higher osteotropism and tumor-homing ability (ADMSC^{Sel} cells).**

The normal adipose tissue derived stem cells (ADMSC^{Cont}) were generously provided by Dr. Jeffery M. Gimble (Pennington Biomedical Research Center, Baton Rouge, LA). We have successfully isolated three different populations with tumor-homing potential. Briefly, ADMSC^{Cont} populations with high tropism towards bone metastatic PC cells (LNCaP) were enriched using an *in vitro* trans-endothelial migration (TEM) system. The human bone marrow endothelial cells (hBMEC-1) barrier (kindly provided by Dr. Graça D. Almeida-Porada, University of Nevada, Reno, NV) cultured onto Matrigel™-coated membrane inserts (8 µm pore size) in 12-well plates to generate a confluent hBMEC-1 barrier on the upper chamber. The permeability of the microvessel barrier was checked with Evans blue dye by colorimetric assays. The conditioned medium of LNCaP cells (CM) was added to the lower chamber. The CM was prepared by growing the LNCaP cells in complete medium (RPMI, 10% FBS 7 antibiotics) to 80% confluency. The medium was then removed and replaced with serum-free RPMI medium for 24 hr. The CM is filtered (0.2 µm) and stored in -20°C until used. The ADMSC^{Cont} cells (1 x 10⁵) were added onto the microvessel barrier and allowed to migrate towards the CM in the lower chamber for 48 hr. Only ASC isolates with tropism towards PC CM (ADMSC^{Sel}) were propagated (passage <3), stored and used in subsequent experiments.

Task-5. Construct a lentiviral vectors (LV) expressing a secretable 3α-HSD enzyme and optimize the LV-transduction and transgene expression in enriched ADMSC^{Sel} cells. Months 14-20

5.1. **Construct the delivery plasmid, by cloning AKR1C4 cDNA into the expression vector pLVX-IRES-ZsGreen1.**

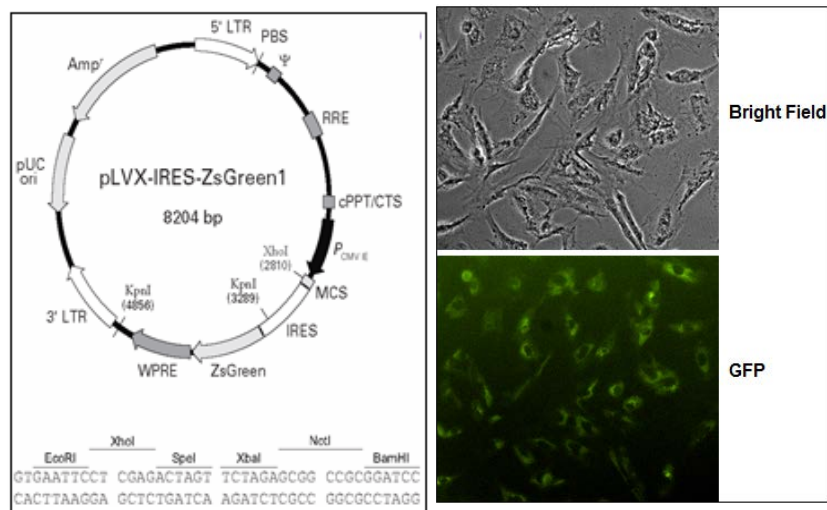
5.2. **PCR amplify the PTD-AKR1C9 region and subclone into pFUSE-mFc2 which contains the 21 amino acid secretory signal (ss) from the IL2 gene.**

Figure-1

Figure-2

In another set of experiments, we purchased a lentivirus construct, pLVX-IRES-ZsGreen1, from Clontech, Inc. (Fig.1). The construct is an HIV-1-based, lentiviral expression vector that allows the

simultaneous expression of protein of interest and ZsGreen1 in virtually any mammalian cell type, including stem cells. ZsGreen1 is a human codon-optimized variant of the reef coral *Zoanthus sp.* GFP, ZsGreen. The vector expresses the two proteins from a bicistronic mRNA transcript, allowing ZsGreen1 to be used as an indicator of transduction efficiency and a marker for selection by flow cytometry. The expression plasmid was used to generate a lentivirus expressing GFP (EF1-alpha GFP lentivirus) at the Applied StemCell, Inc. Briefly, 24 hr before transfection,



Lenti-X 293T cells/100 mm plate were cultured to 80–90% confluency. Next, plasmid and Xfect Polymer solutions were mixed and incubated for 10 min at RT and subsequently added to the cultured cells. After 4

hr, the transfection medium was replaced with fresh complete growth medium and cells were incubated at 37°C for an additional 48 hr. The lentiviral supernatants were pooled, centrifuged and filtered through a 0.45 µm filter to remove cellular debris. Virus production was verified with Lenti-X GoStix™. The EF1-alpha GFP lentivirus virus titer was $2.61 \pm 0.29 \times 10^8$. The viral stocks were aliquoted and stored at -80°C.

The ADMSC^{Cont} cells were transduced with pLV-GFP using a standard protocol. Briefly, 10,000 cells/well were plated in a 24-well plate overnight in DMEM-F12 medium. After washing, the medium was replaced in a fresh phenol-red free medium containing 10 µg polybrene and pLV-GFP and cell were cultured overnight. The medium was changed and transduction efficiency was ~ 90%, as assessed with fluorescence microscope (**Fig. 2**). The GFP-expressing ADMSC^{Cont} cells were further sorted by FACS and the enriched populations were propagated in cultured and stored for future *in vivo* experiments.

5.3. Isolate the fragment coding for IL2ss-HSD and clone into the polylinker site of pShuttle where transgene is under the control of CMV promoter/enhancer to generate pLVX-IL-2SS-Akr1c14-IRES-GFP.

5.4. Remove the CMV enhancer sequences from pEF1-IL2ss-PTD-HSD and insert the Osx binding element (-149 to +27) of human collagen gene (COL11A2) to generate the pOsx-IL2ss-PTD-HSD.

5.5. Cotransfect 293 T-cells with the pseudotyping plasmid, packaging plasmid, and transgene plasmid and harvest LV particles.

The Akr1c14 (NM_138547) rat cDNA clone coding for 3α-hydroxysteroid dehydrogenase (3α-HSD) was obtained from Origene. The 3α-HSD, also known as aldo-keto reductase family 1 member C4, is an enzyme that in humans is encoded by the *AKR1C4* gene. The IL2-SS (signal sequence) was synthesized (IDT) as shown in **Fig. 3**. The IL-2SS enables secretion of Akr1C14 gene production by the ADMSC^{Cont} cells.

Figure-3

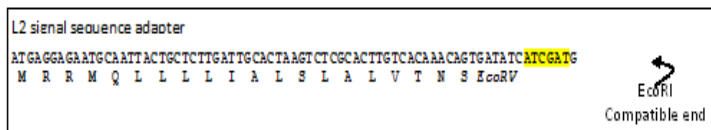
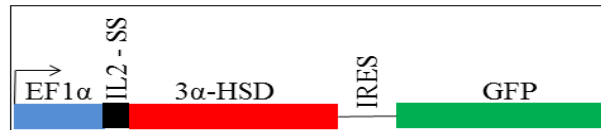


Figure-4



Initially, the Akr1c14 gene was cloned in-frame with IL-2SS at the N-terminus in pCR-II plasmid using EcoRI and EcoRV restriction enzymes and adaptors and the DNA insert was sequence verified. The IL-2SS- Akr1c14 sequence was PCR amplified with SpeI and NotI anchored primers. The PCR product was subcloned in SpeI-NotI digested pLVX-IRES-ZsGreen1 plasmid (pretreated with CIP) to generate pLVX-IL-2SS- Akr1c14-IRES-GFP construct, as shown in **Fig. 4**. The DNA insert was in-frame and was sequenced verified.

We employed the strategy described in Task 4.2 (Applied StemCell, Inc.) to generate lentivirus expressing the pLVX-IL-2SS- Akr1c14-IRES-GFP gene product. The viral titer was $3.26 \pm 0.53 \times 10^8$.

5.6. Transduce ADMSC^{SeI} cells with the LV particles (10-100 MOI) and monitor transgene expression by RT-PCR and its secretion in the media by ELISA assays.

For PCR analysis, total RNA was extracted and cDNA synthesis was performed using standard protocols. from stem cells transduced with control plasmid or the transgene-expressing plasmid. Target genes were amplified with β-actin and Akrc14 specific primers and examined for expression using ethidium bromide stained gels.

Figure-5

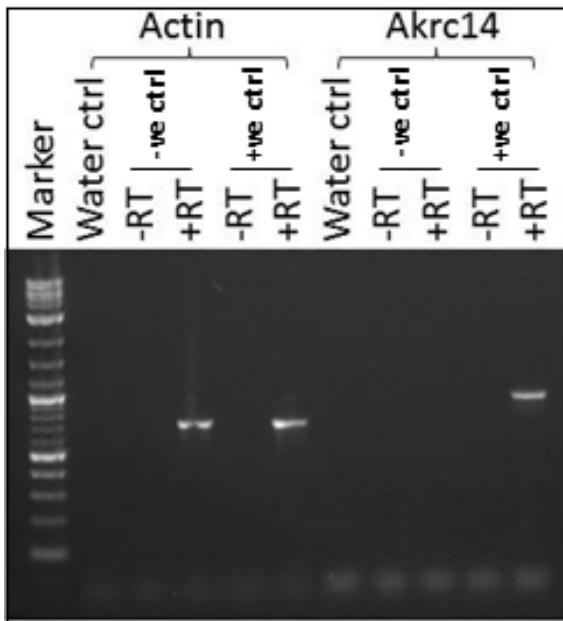
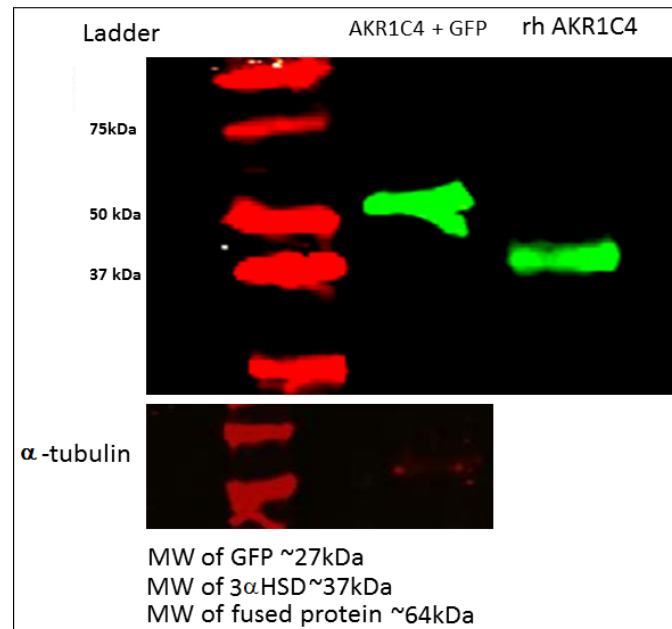


Figure-6



As shown in **Fig. 5**, the transgene (Akr1c4) is expressed in the in stem cells transduced with transgene-expressing pLVX-IL-2SS-Akr1c4-IRES-GFP construct but not by the control or negative controls. The loading controls are shown by expression of actin.

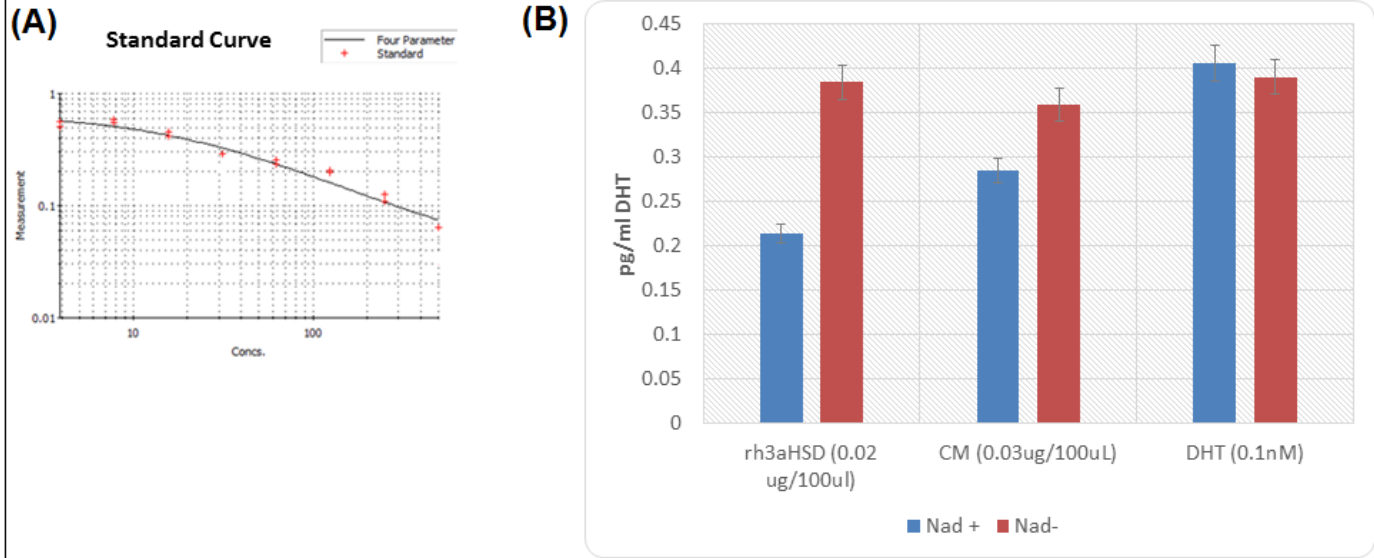
For Western blot analysis, the ADMSC^{Se} were transduced with control plasmid (pLVX-IRES-GFP) or the transgene-expressing pLVX-IL-2SS- Akr1c14-IRES-GFP as shown above and cells were cultured for 48 hr. The medium was collected and examined for transgene expression and release by the stem cells using western blot analysis against rh-3 α SHD. The immunoblot analysis was performed by washing (PBS/Tween20) and blocking (Licor), followed by incubation in primary (1:1,000 dil) and secondary (1/10,000). The membranes were scanned using Licor Odyssey system. As shown in **Fig. 6**, the transduced cells successfully produced the fused transgene (IL-2SS-3 α HSD-GFP) (~64 kDa) as opposed to the rh-3 α HSD alone.

Task-6: Investigate the *in vitro* ability of engineered ADMSC^{SeI} cells to metabolize androgens and suppress tumor cell growth, in CaP and/or in CaP/ADMSC^{AA} cocultures. 20-24

6.1. Monitor functional α HSD expression by pLVX-IL-2SS-Akr1c14-IRES-GFP or the control LV constructs transduced ADMSCs by incubating ADMSC-CM with T or DHT and carry out androgen ELISA assays.

The functional significance of released 3 α HSD by engineered ADMSCs in degrading/inhibiting DHT was explored using Cayman EIA Testosterone kit. Using 96-well plates, the conditioned medium (CM; 30 ng) of the engineered stem cells or the rh3 α HSD (20 ng) were added to the wells in triplicates in presence or DHT (0.1 nM) with or without NAD, required for 3 α HSD activity.

Fig. 7: Testosterone ELISA



Shown in **Fig.7A** is the standard curve of testosterone or DHT ELISA. **Fig. 7B** depicts efficacy of the rh3aHSD in inhibiting DHT by approximately 50% at 20 ng concentration in presence, but not absence of NAD. Likewise, though to a lesser extent, the CM of engineered cells was able to suppress DHT by approximately 20% in presence of NAD at 30 ng concentration. The experiment, is preliminary, and would be repeated to optimize the amount of CM (a range of 20 to 100 ng) to achieve more potent inhibition of DHT by the transgene *in vitro*.

In a separate set of reporter assay experiments, we have examined the efficacy of CM of engineered stem cells in suppressing DHT induced AR transactivation in androgen dependent LNCaP cells. In these experiments we increased the concentration of CM to 80 ng based on weak response of 20 ng CM shown in Fig. 7B. LNCaP cells were transiently transfected with a psPSA-luc plasmid, encompassing a PSA truncated promoter with AREs, or the control pcDNA3.1 plasmid, for 24 hrs. Cells were then treated conditioned media (CM) of engineered stem cells in presence or absence of DHT and NAD. As shown in

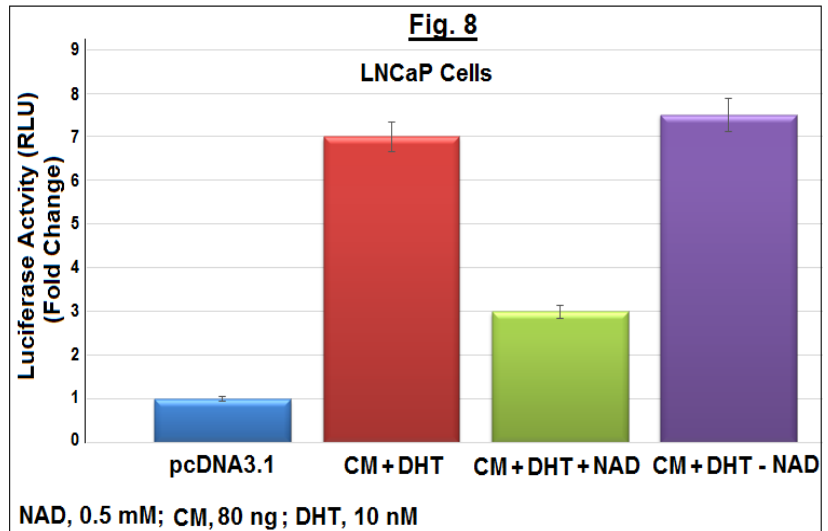
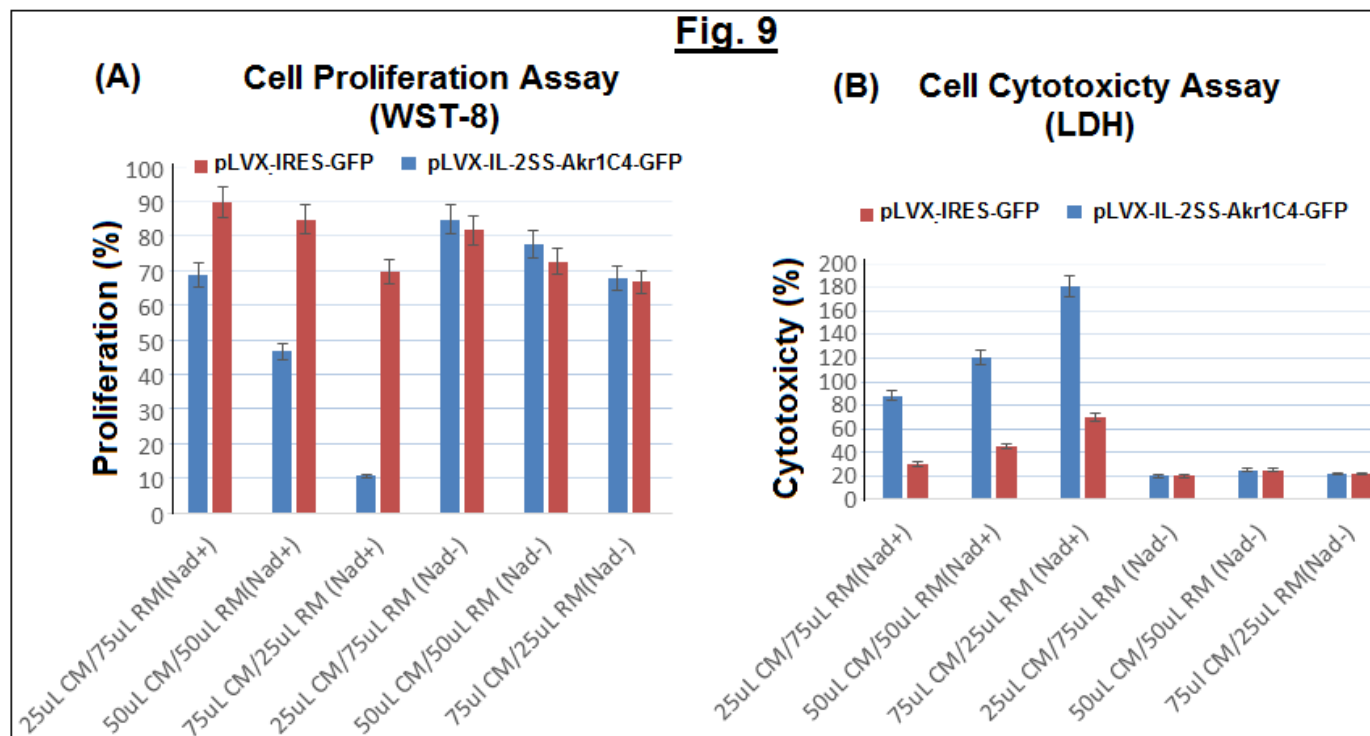


Fig. 8, the DHT/AR activity was significantly suppressed (>50%) by CM in presence of NAD, suggesting it is effective at this or higher concentration to degrade/suppress DHT in the tumor microenvironment.

6.2. Coculture the GFP-labeled CaP cells with either control ADMSC^{SeI} or engineered ADMSC^{SeI} cells and compare growth rates in tumor cells, similar to Task-2.4.

6.3. Coculture the GFP-labeled CaP cells with ADMSC^{AA} cells similar to Task-2.4., and equal numbers of control ADMSC^{SeI} or engineered ADMSC^{SeI} cells and compare growth rates in tumor cells.



Next, we examined the ability of the conditioned medium (CM) of the enriched engineered stem cells (ADMSC^{SeI}) in inhibiting the growth and induce apoptosis of the androgen-dependent LNCaP cells *in vitro*. The cells were maintained to 50% confluency in 96-well plates in regular medium (RM) encompassing RPMI supplemented with 10%FBS. The medium was replaced with CM derived from ADMSCs transduced with control viral construct or Akr1C4 expressing construct containing various concentrations of CM (25 μ L, 20 ng; 50 μ L or 75 μ L, 80 ng) in presence or absence of NAD. As shown in **Fig 9A**, the CM of Akr1C4-expressing cells showed a growth inhibition in a dose-dependent manner in presence of NAD, whereas no effect was observed in absence of NAD. The results suggest that 3 α HSD was effective in inhibiting the growth of LNCaP cells through degradation of hormone in the medium. The results were corroborated by LDH cytotoxicity assays, demonstrating increased cell death by 3 α HSD-containing medium in comparison to control media (**Fig. 9B**).

Discussion

Emerging evidence suggests that “intracrine” androgens, produced *de novo* in the tumor microenvironment, pose challenge in the clinical management of prostate cancer. A number of studies demonstrated that the levels residual androgens at metastatic sites of castrated patients are sufficient to restore AR signaling and support growth of prostate tumor cells. To overcome, the therapeutic limitation of the androgen ablation therapy, we proposed to examine the efficacy of androgen catabolizing enzyme, 3 α HSD, in degrading/suppressing DHT activity in the tumor microenvironment. In our proof-of-concept experiments, presented herein, we were able to construct in a lentivirus plasmid, pLVX-IRES-ZsGreen, expressing secretable IL-2SS-Akr1C4 gene product. Also, we successfully packaged and produced both control GFP-, and IL-2SS-Akr1C4-expressing lentiviral constructs in high titers and transduction efficiency of normal stem cells with tumor homing-potential (ADMSC^{SeI}). The expression of the transgene (3 α HSD)

was verified by PCR and Western blot analysis. Subsequent functional assays demonstrate that 3 α HSD (whether recombinant or produced in CM) is effective in degrading DHT *in vitro*. The suppression of DHT activity is coupled by inhibition of AR transactivation and proliferation and induction of cytotoxicity of the androgen-dependent LNCaP cells. Together, our findings propose new concepts for targeted residual androgens within the tumor microenvironment to circumvent clonal expansion at primary and metastatic sites. Our observations also suggest that a multi-targeted hormonal therapeutic modality designed to ablate 'intracrine' androgens by pASCs is required to enable effective and optimal treatment response in patients with castration-resistant PC (CRPC), especially among AA-men.

Key Research Accomplishments: The following experiments were completed:

- a. Using transendothelial well culture (TWC) chambers, we successfully isolated and enriched ADMSC^{Cont} with homing potential towards conditioned medium (CM) of prostate cancer cells (ADMSC^{Sel}).
- b. Constructed a plasmid expressing IL-2SS-AKR1C4 cDNA into the expression vector pLVX-IRES-ZsGreen1.
- c. Generated lentiviral vectors (LV) expressing GFP or a secretable 3 α -HSD enzyme and optimize the LV-transduction and transgene expression in enriched ADMSC^{Sel} cells.
- d. Generated ADMSC^{Sel} clones expressing GFP (control) or the target gene (IL-2SS-AKR1C4) for the proposed *in vitro* and *in vivo* studies.
- e. The release of the transgene by ADMSC^{Sel} was verified by PCR and Western blot analysis.
- f. The 3 α HSD (whether recombinant or produced in CM) is effective in degrading DHT only in presence of NAD *in vitro* (ELISA assays).
- g. The suppression of DHT activity is coupled by:
 - (i) Inhibition of AR transactivation (reporter assays).
 - (ii) Inhibition of the androgen-dependent LNCaP cell proliferation *in vitro* (WST-8 assays).
 - (iii) Induction of cytotoxicity (cell death) of LNCaP cells *in vitro* (LDH assays).

Reportable Outcomes:

1. Meeting presentations: The outcome of the studies will be presented at the annual AACR meeting in Washington, DC, 2014.
2. Manuscript is under preparation for publication in the foreseeable future.

Conclusions: African Americans (AA) have twice the incidence and mortality of prostate (PC) than Caucasian Americans (CA). While the disproportionate burden was partially explained by genetic, socioeconomic, and environmental factors, racial variation in the biology of prostate tumors was not investigated. Emerging evidence suggests that unknown factors promote tumor growth via unknown mechanisms. Recently, "Intracrine" androgens have been consistently implicated in the outgrowth of castration-adapted prostate tumors through activation of functional androgen-signaling cascade under androgen deprivation therapy (ADT). To overcome, the therapeutic limitation of the androgen ablation therapy, we proposed to examine the efficacy of androgen catabolizing enzyme, 3 α HSD, in degrading/suppressing DHT activity in the tumor microenvironment. In our proof-of-concept experiments, presented herein, we were able to construct in a lentivirus plasmid, pLVX-IRES-ZsGreen, expressing secretable IL-2SS-Akr1C4 gene product. Also, we successfully packaged and produced both control GFP-, and IL-2SS-Akr1C4-expressing lentiviral constructs in high titers and transduction efficiency of normal stem cells with tumor homing-potential (ADMSC^{Sel}). The expression of the transgene (3 α HSD) was verified by PCR and Western blot analysis. Subsequent functional assays demonstrate that 3 α HSD (whether recombinant or produced in CM) is effective in degrading DHT *in vitro*. The suppression of DHT activity is

coupled by inhibition of AR transactivation and proliferation and induction of cytotoxicity of the androgen-dependent LNCaP cells. Together, our findings propose new concepts for targeted residual androgens within the tumor microenvironment to circumvent clonal expansion at primary and metastatic sites. Our observations also suggest that a multi-targeted hormonal therapeutic modality designed to ablate *'intracrine'* androgens by pASCs is required to enable effective and optimal treatment response in patients with castration-resistant PC (CRPC), especially among AA-men.

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