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

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 \textit{(intracrine)} 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. 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. This will be achieved by: (a) investigate if ‘intracrine’ production of testosterone by osteotropic ASC\textsuperscript{AA} modulates growth and metastatic potential of CaP cells under ADT in vitro and in vivo; (b) determine if α-HSD-expressing osteotropic ASC\textsuperscript{Cont} will nullify the ADMSCAA-mediated CaP cell growth and metastasis in vitro; and (c) examine the efficacy of therapeutically engineered ASC\textsuperscript{Cont} to target and inhibit CaP tumor growth under CRPC \textit{in vivo}. The proposed work will be \textit{innovative}, 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.

Prostate cancer, health disparity, stem cells, hormone inactivating enzymes, CRPC, steroidogenic enzymes, African Americans.
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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

Initially, we confirmed Data generated last year in Aim-2. To this end, the optimal dose, growth inhibitory effects, and ability of recombinant α-HSD to degrade DHT and suppress AR activity in LNCaP cells were examined in vitro. Briefly, the conditioned media (CM) of normal ADMSCs transduced with either pLVX-IL-2SS-Akr1c14-IRES-GFP or the control LV constructs were collected and protein concentrations were measured by BCA assays. Figure 1 (next page) depicts high expression of Akr1c14-
IRES-GFP (α-hydroxysteroid dehydrogenase, α-HSD) and GFP in tumor-tropic ADMSCs. The enzyme gene expression and the release of its recombinant protein product were measured by PCR and immunoblot analysis (Fig. 1).

Next, we examined the effect of the enzyme in the CM on growth of the androgen dependent LNCaP cells. Briefly, cells were cultured overnight in a charcoal stripped medium supplemented with 0.1 nM DHT in 96-well plates in triplicates and then subjected to various protein concentrations of CM derived from control or 3αHSD-expressing ADMSCs for an additional 24 hrs. As shown in Figure 2, the enzyme inhibited growth of the LNCaP cells in a dose dependent manner in presence, but not in absence, of NAD, which is required for enzyme activity. The growth inhibition/cell death was monitored with MTT (Fig 2A) and LDH (Fig 2B).

To verify if the growth inhibitory and induced cell death is mediated through degradation of androgens, DHT levels were measured by EIA assay in LNCaP cells treated with rhAKR1C4 or CM derived from control or 3αHSD-expressing ADMSCs. As shown in Figure 3, both rhAKR1C4 and r3αHSD degraded DHT in presence NAD compared to controls (Fig 3A). Additionally, the degradation of in the medium was coupled with suppression of AR activity in LNCaP cells (Fig 3 B). The results suggested that the enzyme-induced growth inhibition/cell death in LNCaP cells was attributed to degradation of DHT and suppression of AR activation in the androgen-dependent cells.

In another set of in vitro experiments, we tested the hypothesis that tumor-tropic CaP patient-derived, but not normal, contributes to production of residual androgens upon recruitment and interaction with prostate tumor cells in primary or metastatic microenvironment. To achieve this, we examined and compared if conditioned media (CM) of prostate cancer (CaP) cells induces expression of androgen metabolizing enzymes (AMEs) in tumor-tropic enriched ADMSCs procured from Gleason score-matched (Gleason 6) African Americans (AA) and Caucasian (CA) prostate cancer patients. Briefly, normal and AA-derived ADMSCs were cultured in DMEM F-12 with 10% charcoal stripped FBS and at 70% confluence,
and then treated with CM of LNCaP, C4-2B or normal prostate epithelial cells (RWPE1) for 72 hours. Thereafter, AME genes expression was quantitated through qRT-PCR and the results were normalized to normal stem cells. The results shown in Figure 4, demonstrate that the CaP cell CM-treated ADMSCs from AA-men, but not normal ADMSCs, induces steroidogenic gene expression of 17-β-hydroxysteroid dehydrogenase (17beta-HSD) as well as SRD5A1 and 2 transcripts, which encode for 5α-reductase subtype 1 and 2 required for conversion of T into dihydrotestosterone (DHT) in response CM of LNCaP and C4-2B cells, and a decrease in androgen hydrolyzing enzyme 3αHSD1 compared to CM of normal prostate epithelial cells (RWPE1). The results suggest that tumor-tropic ADMSCs contributes to production of residual androgens in the tumor microenvironments (primary or metastatic) in prostate cancer patients, especially among AA-men. Our initial analysis of AME expression in 5 ADMSCs derived from CA men (data not shown) demonstrate lesser expression of AMEs compared to ADMSCs derived from AA men, further attest to racial differences in production of residual androgens in the tumor microenvironment.

We further examined if there are differences in androgen dependent prostate tumor growth in vivo due to racial disparity of residual androgen production by CaP patient-derived ADMSCs from AA and CA men. Briefly, LNCaP cells (2 x 10^6) were mixed in Matrigel with ADMSCs (5 x 10^5) procured from AA (n=5) or CA (n=5) with CaP and inoculated s.c. on the right and left flank of athymic nude mice, respectively. Tumor growth was monitored for 6 weeks. As shown in Fig. 4B, LNCaP tumor formation was larger (2 to 3 fold) when co-inoculated with ADMSCs from AA (right flanks) compared to ADMSCs from CA men with CaP. The results further affirm that production of residual androgens by ADMSCs in the tumor microenvironment may contribute to disease progression among AA men. Current studies are focused on immune-staining of key AME enzymes by IHC on tumor sections. A manuscript under preparation.

Figure: 4B

In addition to the above experiments, we attempted to complete Specific Aim 3 in the 3rd year of the project as described below:

**Specific Aim-3:-** Examine the efficacy of the therapeutically engineered ADMSC\(^{\text{Cont}}\) to target and inhibit CaP tumor cell growth under hormone deprived conditions in vivo. This aim tests the working hypothesis that therapeutic targeting by osteotropic α-HSD-expressing ADMSC\(^{\text{Cont}}\) suppresses CaP cell metastatic lesions when co-transplanted in bone with ADMSC\(^{\text{AA}}\) in castrated male mice.
Task-3-1: Compare the ability of ADMSC\textsuperscript{Cont} and ADMSC\textsuperscript{Sel} cells to colocalize to bone tumor xenografts \textit{in vivo}. 24-30

3.1.1 Inject CaP cells, alone or with ADMSC\textsuperscript{AA}, at intraosseous (femur) sites in nude-mice and monitor tumor development by tumor (~3-5 wks) by palpating and by serum PSA levels.

Unlike soft tissue, current imaging technologies are limited in their ability to detect tumor formation in bones. To overcome such a limitation, we employed IRDye® 800CW BoneTag™ Optical Probe (LI-COR); calcium-chelating compounds have been used effectively for the detection of bone mineralization, to produce a near infrared (NIR) optical bone marker for small animal imaging. To perform the \textit{in vivo} testing, LNCaP cells (2 x 10\textsuperscript{6}) were re-suspended in Matrigel and injected intra-tibially in in 3 nu/nu mice. After 4 to 6 weeks, the IRDye IRDye 800 BoneTag optical probe (reconstituted in 0.5 ml of sterile 1X PBS for a final concentration of 0.02 nmol/μL) was injected at 2 nmol (100 μL) intravenously via the tail vein. Imaging was performed 48 hrs later to allow unretained agent to clear from the animal’s circulation. I imaging was performed on sedated mice using Odyssey CLX at 800 channel by placing the animal on the imaging bed with the tumor side facing down for optimal signal. As shown in Figure 5 (previous page), tumors successfully developed in the left tibia (white arrow) in 2 out of 3 animals tested and their images was captured by Odyssey system. However, a limitation of this system is that the tumor size cannot be quantitatively measured. To overcome this problem, we plan to use RDye 800CW 2-DG Optical Probe (Catalog# 926-08946), which specifically stain tumor cells green, or the Y-27 Optical Probe specifically designed to target prostate specific membrane antigen (PSMA) on tumor cells. These studies are currently pursued (see below).

3.1.2. Tail vein injection of labeled-ADMSCs (either ADMSC\textsuperscript{Cont} or ADMSC\textsuperscript{Sel} cells from same batch) and monitor whether the enriched ADMSCs cells preferentially engraft within the bone tumor foci. These experiments are meant to monitor the ability of LV-transduced ADMSC\textsuperscript{Sel} cells to suppress the CaP cell growth promoting effects of ADMSC\textsuperscript{AA} cells in tumor xenografts \textit{in vivo}. The completion of these experiments is contingent upon successful transplantation of LNCaP in bone (Aim 3.1.1).

3.1.3. Investigate the effects of coinjected ADMSC\textsuperscript{Sel} cells producing α-HSD on tumor size in male nude-mice subcutaneously injected with CaP cells.

In a pilot study, we examined the efficacy of dual labeling (green for tumor and red for bone) in nude mice. To achieve this goal, three nude mice were injected s.c. and intra-tibially with LNCaP cells as shown above. This was followed by iv injection of IRDye 680RD Optical Probe, which stained tumors with green color, and IRDye 680RD, which stain bone with red color. As shown in Fig 6, the LNCaP tumors stained green and the bone stained red. While s.c tumors are well developed, the intra-tibial tumors are weekly stained with green (yellow circle). Additionally, the guts stained non-specifically with the bone dye due to presence of minerals in the diet (red arrow). Due to poor intra-osseous tumor growth, the results
suggest that the s.c. model will be suitable to trace engraftment and expression of the transgene by the tumor-tropic ADMSCs at the tumor sites in vivo (Fig 6).

We requested a one-year extension to use the developed models to (a) determine the effects of engineered ADMSC$^{\text{Sel}}$ cells injected via tail vein on growth of bone tumor xenografts containing CaP cells, alone or with ADMSC$^{\text{AA}}$; (b) determine ADMSCs (mCherry) engraftment and local α-HSD expression in tumor bearing mice injected with either ADMSC$^{\text{Cont}}$, ADMSC$^{\text{Sel}}$ or engineered ADMSC$^{\text{Sel}}$ cells; (c) carry out similar experiments in castrated mice, to demonstrate that the tumor-homing of engineered ADMSC$^{\text{Sel}}$ cells can target local androgens and suppress tumor growth, even under ADT.
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, 3αHSD, in degrading/suppressing DHT activity in the tumor microenvironment. In studies presented herein, we were able to generate stable normal ADMSCs expressing GFP using a lentivirus construct. Additionally, normal ADMSCs transduced with a pLVX-IRES-ZsGreen construct successfully expressed secretable IL-2SS-Akr1C4 gene product. The secreted Akr1C4 successfully inhibited growth of androgen-dependent LNCaP cells in vitro. Subsequent functional assays demonstrate that 3αHSD (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. Additionally, after several attempts, we also demonstrated using bone tag probes that LNCaP tumors could be successfully developed in long bone through intra-tibial injections. However, current strategies are focused on use of dual fluorescent color system to make osseous tumor growth quantifiable. Additionally, a model based on s.c. transplantation was also established to quantify targeting of ADMSCs and tumor growth in vivo. In another set of experiments, we demonstrate that, unlike normal ADMSCs, patient derived ADMSCs express AMEs in response to the CM of prostate cancer cells. Moreover, ADMSCs from African Americans (AA-men) have higher AME expression than those procured from Caucasian Americans CA). Co-transplantation of ADMSCs from AA-men with LNCaP cells triggered 2 to 3 fold higher tumor growth in comparison to CA-derived ADMSCs in vivo. 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 in tumor environment 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. The recombinant 3αHSD (whether recombinant or produced in CM) is effective in degrading DHT only in presence of NAD in vitro (ELISA assays).

b. The recombinant, whether recombinant or produced in CM, was able to inhibit growth and AR transactivation in androgen-dependent LNCaP cells in a dose dependent manner.

c. Patient-derived ADMSCs, especially from AA-men, express AMEs and DHT when triggered by CM of prostate cancer cells compared to CA-men.

d. The patient-derived ADMSCs from AA-men trigger prostate tumor growth by 2 to 3 fold when co-transplanted with LNCaP cells compared to CA-derived ADMSCs.

e. LNCaP tumors successfully developed intraosseously. The tumors were detected by Li-COR using bone tag probes. Dual fluorescent probes are currently tested to quantify engraftment of ADMSCs and inhibition of tumor growth by 3αHSD-expressing ADMSCs.

f. We developed a s.c. LNCaP tumor xenografts as alternative strategy to bone xenografts.

Reportable Outcomes

A manuscript will be submitted for publication.
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, 3αHSD, in degrading/suppressing DHT activity in the tumor microenvironment. In studies presented herein, we were able to generate stable normal ADMSCs expressing GFP using a lentivirus construct. Additionally, normal ADMSCs transduced with a pLVX-IRES-ZsGreen construct successfully expressed secreteable IL-2SS-Akr1C4 gene product. The secreted Akr1C4 successfully inhibited growth of androgen-dependent LNCaP cells in vitro. Subsequent functional assays demonstrate that 3αHSD (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. Additionally, after several attempts, we also demonstrated using bone tag probes that LNCaP tumors could be successfully developed in long bone through intra-tibial injections. However, current strategies are focused on use of dual fluorescent color system to make osseous tumor growth quantifiable. Additionally, a model based on s.c. transplantation was also established to quantify targeting of ADMSCs and tumor growth in vivo. In another set of experiments, we demonstrate that, unlike normal ADMSCs, patient derived ADMSCs express AMEs in response to the CM of prostate cancer cells. Moreover, ADMSCs from African Americans (AA-men) have higher AME expression than those procured from Caucasian Americans CA). Co-transplantation of ADMSCs from AA-men with LNCaP cells triggered 2 to 3 fold higher tumor growth in comparison to CA-derived ADMSCs in vivo. 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 patient derived ADMSCs is required to enable effective and optimal treatment response in patients with castration-resistant PC (CRPC), especially among AA-men.


