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TITLE: Androgen Deprivation Therapy Resistance, Fatty Acid Oxidation, and the Role of Beta Hydroxybutyrate

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14. ABSTRACT For prostate cancer (PCa), androgen deprivation therapy (ADT) (eg. abiraterone acetate and enzalutamide) is the standard systemic treatment. Although initially effective, within 2-3 years of treatment, most patients will develop castration resistant prostate cancer (CRPC). Although mechanisms that promote CRPC are uncertain, it is noted that ADT confers a selective pressure. While most research has focused on PCa epithelia,our lab has shown that there is a selective pressure over the surrounding stroma as well. In essence, these fibroblasts co-evolve to support tumor growth under adverse conditions. Regulation of fatty acid oxidation (FAO)has been identified as a major source of energy in PCa. In fact, clinical studies have shown that enzymes involved in the ketogenic pathway are increased in PCa progression. Interestingly, we found that ketone body production was increased in fibroblasts when exposed to ADT, while its utilization by adjacent epithelia was promoted. Ketone bodies like beta-hydroxybutyrate (BHB) serve as a source of energy but there is ongoing evidence of its importance as a signaling molecule, HDAC inhibitor and histone modifier. Given that BHB can have an important pharmacological role in cancer, we sought to investigate the role of this molecule in CRPC. Preliminary data from our lab shows that ADT non-responsive patients have lower levels of BHB in serum which was in concert with the observed elevated BHB utilization by epithelia in culture. Therefore, we hypothesize that FAO modulation through ADT promotes CRPC phenotype. Blockade of fatty acid metabolism in stroma can sensitize PCa epithelial cells to enzalutamide. Our broad objective is to elucidate mechanisms that promote CRPC by studying the role of FAO in stromal-epithelia interaction.

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Introduction

PCa is the most commonly diagnosed non-cutaneous type of cancer, and the second leading cause of death (1). Androgen signaling deprivation therapy (ADT) (*e.g.* abiraterone acetate and enzalutamide) is the standard systemic treatment. Although ADT is initially effective, within 2-3 years of treatment, most men will progress towards castration resistance (CRPC). Unfortunately, management for metastatic CRPC involves adjuvant therapy with taxane-derivative drugs (*e.g.* docetaxel) with poor subsequent survival (2,3). It is noted that ADT therapy confers a selective pressure that changes the epithelial cells. Some changes include, androgen receptor (AR) reactivation via over expression, enzyme up-regulation and the expression of aberrant splice variants (4). Although most research has focused PCa epithelia, our lab has shown that there is a selective pressure over the surrounding stroma as well. In essence, fibroblasts co-evolve to support tumor growth under adverse conditions like ADT or nutrient deprivation (5). Data from our lab shows a genetic signature that can differentiate tumor-inductive stroma (cancer associated fibroblasts; CAF) - and non-tumor inductive stroma (normal associated fibroblasts; NAF) (6). In addition, we show that fibroblast exposure to ADT (*e.g.* castration, abiraterone, enzalutamide) promotes a CAF-like genetic signature. Regulation of fatty acid oxidation (FAO) has been identified as a hallmark of cancer. For PCa epithelia, unlike many other Warburg (glycolysis-dependent) cancers, FAO is a major source of energy. Moreover, there is clinical evidence that enzymes involved in the ketogenic pathway are increased in PCa progression (7). Ketone bodies like beta-hydroxybutyrate (BHB) serve as a source of energy during starvation. In addition to being a substrate for metabolic breakdown, BHB is also a recognized signaling molecule, HDAC inhibitor, and histone modifier (8,9). Given the growing evidence that BHB can have multiple roles in cancer, we sought to investigate its impact in CRPC.

Keywords

Prostate cancer, cancer-associated fibroblasts, neuroendocrine differentiation, enzalutamide, castration-resistant prostate cancer, androgen deprivation therapy, fatty acid metabolism, ketone body metabolism, beta hydroxy butyrate

Accomplishments

Major goals of the project as stated in the approved SOW

Major Goal 1: Metabolic characterization of NAF and CAF

Major Goal 2: Metabolic profiling of stromal cells when exposed to ADT

Major Goal 3: To define the role of BHB in CRPC

Aim 1. To delineate the role of fatty acid oxidation in NAF and CAF.

Aim 1A. Metabolic characterization of NAF and CAF.

Major Activities: To understand the metabolic characteristics of patient derived NAF and CAF we evaluated the expression of ketone body metabolism genes 3-Hydroxybutyrate Dehydrogenase 1 (BDH1), which catalyzes the reversible conversion between acetoacetate and beta-hydroxy butyrate (BHB), and 3-Hydroxy-3-Methylglutaryl-CoA Synthase 2 (HMGCS2) which catalyzes the rate limiting step. **Specific objectives**: To mimic stress conditions of the tumor microenvironment, we exposed cells to normoxia or hypoxia for 72 hours. **Key Outcomes:** We observed that in hypoxic conditions, CAF have higher expression of HMGCS2. In NAF however, we observed that HMGCS2 expression was negligible in hypoxic conditions. This result suggests that BHB production is promoted in CAF during stress. BDH1 expression was dramatically reduced in NAF during hypoxia while in CAF it remained unchanged (Figure1).

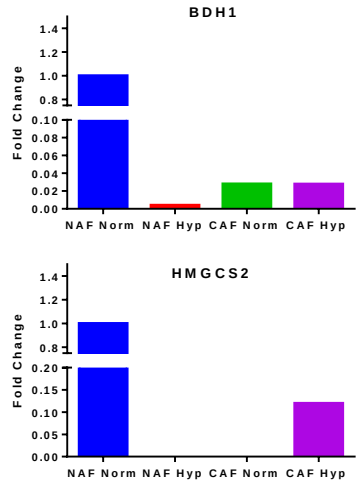

Figure 1: Real-Time PCR results of BDH1 and HMGCS2 expression in NAF and CAF. Fold change calculated using the Ct method relative to NAF under normoxia.

Aim 1B. Interrogate the impact of stromal ketogenesis on epithelia.

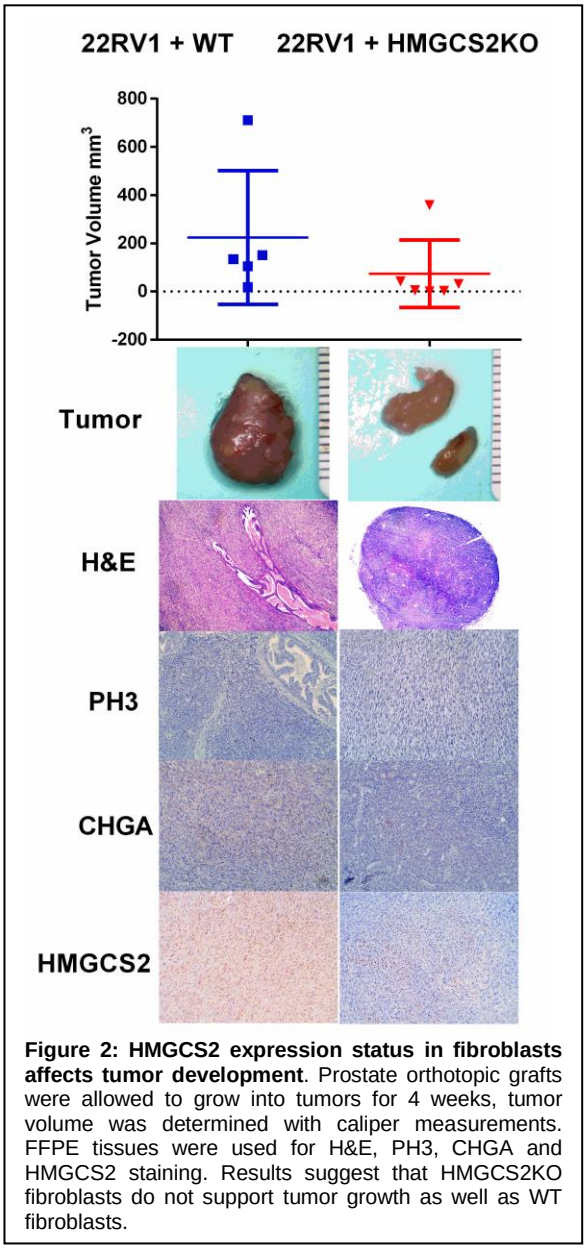

Figure 2: HMGCS2 expression status in fibroblasts affects tumor development. Prostate orthotopic grafts were allowed to grow into tumors for 4 weeks, tumor volume was determined with caliper measurements. FFPE tissues were used for H&E, PH3, CHGA and HMGCS2 staining. Results suggest that HMGCS2KO fibroblasts do not support tumor growth as well as WT fibroblasts.

Specific objectives: To further understand the effect of ketogenesis in tumor growth we performed an *in vivo* experiment. We utilized orthotopic grafting of 22RV1 with either WT murine fibroblasts or HMGCS2 KO fibroblasts. **Key Outcomes:** Our findings, although not significant, show a trend that WT fibroblasts are able sustain to tumor growth better than HMGCS2 KO fibroblasts. Moreover, we observed the same trend with phosphorylated histone H3 (PH3) and chromogranin A (CHGA) (Figure 2).

Aim 1C. Determine if ketogenic differences in PCa patients predict biochemical recurrence following prostatectomy.
Subtask 4: Statistical analysis BHB and Biochemical recurrence
Nothing to report

Aim 2. To determine the action of BHB in CRPC.

Major Goal 2: Metabolic profiling of stromal cells when exposed to ADT
Subtask 1: Seahorse XF OCR and ECAR for stromal cells exposed to ADT
Subtask 2: Generate HMGCS2KO Stromal cells

Aim 2A. Metabolic reprogramming of stromal fibroblasts induced by ADT

Major Activities: To study the effects of ADT in stromal metabolic reprogramming, we exposed murine prostate fibroblasts to enzalutamide for 72h in normoxic and hypoxic conditions. **Key Outcomes:** Our results showed that regardless of oxygen levels, enzalutamide was able to increase the expression of CPT1, CPT2 and HMGCS2 suggesting higher fatty acid oxidation and ketone body production. To discard if ketone body production could cause epigenetic changes, we evaluated Sirtuin 1 (SIRT1) which is a commonly known cell regulator involved in histone deacetylation. However, we did not observe any significant changes. Afterwards, we evaluated mitochondrial turnover using fibroblasts expressing MitoTimer. This tool is a mitochondrial tagged reporter protein (dsRed) which shifts from green to red as it oxidizes. **Key Outcomes:** Our data showed that exposure to enzalutamide enhanced mitochondrial biogenesis, in addition we verified that PGC1a expression was increased using qPCR. Looking at oxygen consumption rate (OCR) using Seahorse we found that overall fibroblasts exposed to enzalutamide have more active mitochondria, we paired this result to the higher expression of CPT1 by qPCR. In addition to this data we looked at the expression of the enzymes involved in ketone body synthesis HMGCS2 and BDH1 and just as we saw with CAF under hypoxia, expression of both these genes was increased. Finally, we evaluated the

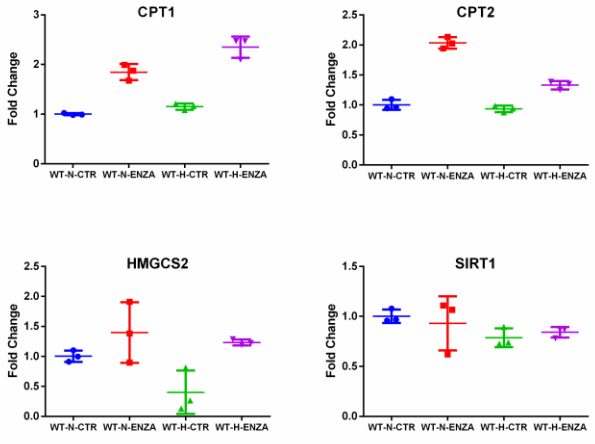

Figure 3: Changes in fatty acid oxidation induced by Enzalutamide. Murine fibroblasts were exposed to 10uM Enzalutamide for 72h in both normoxia and hypoxia. The expression of genes in volved in fatty acid oxidation are significantly increased.

expression of a curated CAF signature panel we have in our lab. We observed that the majority of these were increased, establishing that exposure to enzalutamide can reprogram murine fibroblasts to express more CAF-like characteristics (Figure 4).

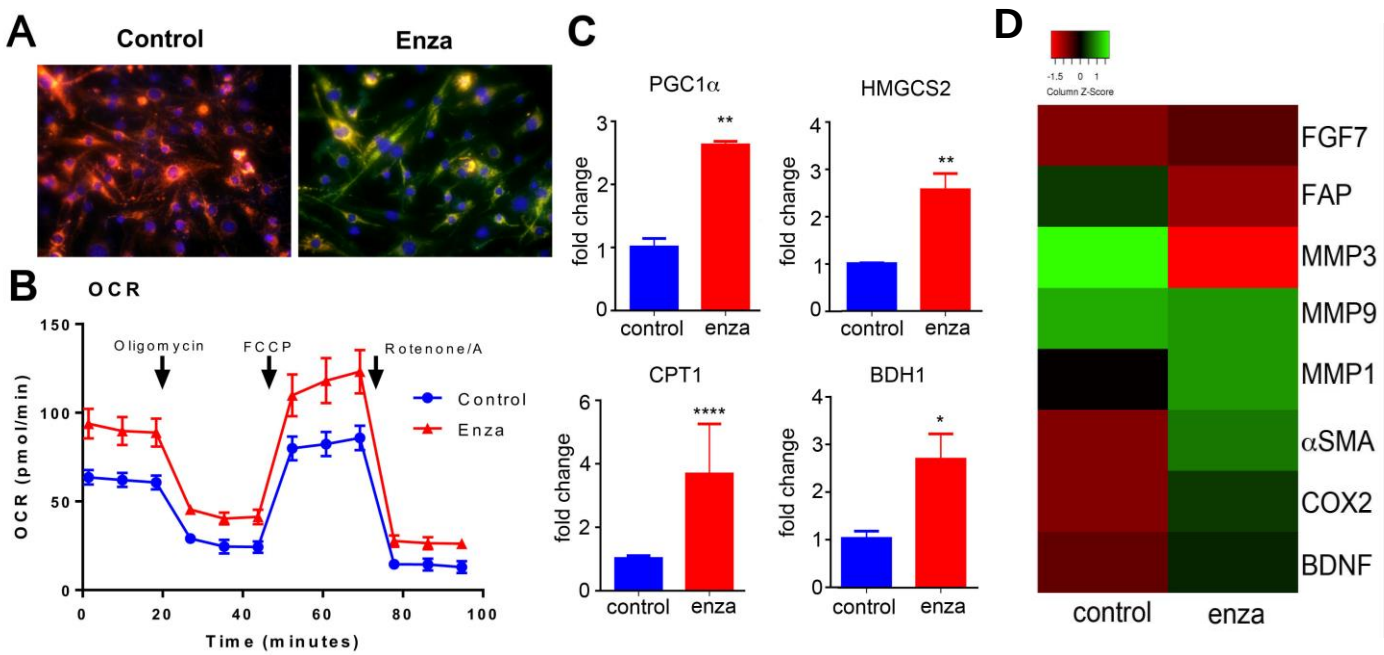

Figure 4: Stromal metabolic changes under ADT. In all cases murine prostatic stroma was cultured to 70% confluency and exposed to enza for 72h. **A)** MitoTimer stroma shows increased mitochondrial turnover (green) when exposed to enza. **B)** Seahorse experiment shows that enza increased basal respiration, spare respiratory capacity, proton leak and ATP production. **C)** qPCR results show increased PGC1a, CPT1, HMGCS2 and BDH1. *P < 0.05, **P < 0.01, ****P < 0.0001 **D)** Expression of curated CAF markers was measured using qPCR and analyzed using the CT method. Fold change is represented in heat map form.

Aim 2B. To define the role of BHB in CRPC.

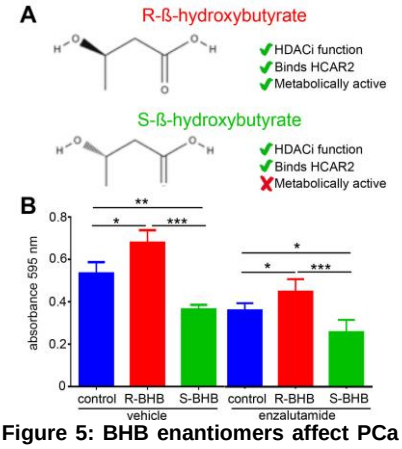

Figure 5: BHB enantiomers affect PCa cell growth and response to ADT A) R-BHB and S-BHB chemical structure. B) 22Rv1 cells were exposed to R or S-BHB for 72 hours in the context of enza 5uM. Growth was significantly reduced with S-BHB while R-BHB rescued survival. *P<0.05, **P<0.01, ***P<0.0001.

Major Activities: To study the effect of BHB in epithelia we first focused on the effects of BHB itself. Given that BHB is a chiral molecule, we took advantage that S-BHB is not readily metabolized but exerts all signaling functions to look at a scenario where BHB is not consumed. **Specific objectives:** For these experiments we cultured 22Rv1 cells with either R-BHB and S-BHB (5mM) and treated them for 72h with Enzalutamide (5uM). **Key Outcomes:** Our results showed that S-BHB was able to increase cell death by Enzalutamide while R-BHB was able to promote survival (Figure 5). This result suggests that S-BHB may have pharmacological value..

Opportunities for training and professional development

The specific goals of this project have increased my knowledge in the use of CRISPR and had raised the opportunity to train other laboratory members in this technique. I have attended scientific meetings like the SBUR which are focused on urologic cancers to share my findings. In addition, with my mentors, we actively discuss career goals and future endeavors in weekly metings.

How were the results disseminated to communities of interest?

These results have been shared in the SBUR meeting as a poster presentation.

What do you plan to do during the next reporting period to accomplish the goals?

During next reporting period we will focus on completing the following goals as approved in the SOW

Major Goal 1: Metabolic characterization of NAF and CAF

Subtask 4: Statistical analysis BHB and Biochemical recurrence

Major Goal 2: Metabolic profiling of stromal cells when exposed to ADT

Subtask 3: Determine ketone body production

Major Goal 3: To define the role of BHB in CRPC

Subtask 1: Generate OXCT1KO PCa cells
Subtask 2: Tissue recombination experiments
Subtask 3: Mouse model, tissue processing and staining

Impact:

What was the impact on the development of the principal discipline(s) of the project?

Our results for the metabolic behavior of NAF and CAF bring more light into the stromal component of the tumor microenvironment and their role in shaping response to therapy. Specifically, metabolic enzymes can be novel targets to improve current therapy and prevent resistance.

What was the impact on other disciplines?

Our findings surrounding the study of ketone body metabolism and thinking of these enzymes as possible targets can expand into other disciplines outside of prostate cancer and currently, we are working on exploring these aspects in other cancer models.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

Changes/Problems

Major Goal 1: Metabolic characterization of NAF and CAF

Subtask 1: Seahorse XF OCR and ECAR for NAF and CAF
Subtask 2: Determine ketone body production in NAF and CAF
Subtask 3: Generate HMGCS2KO stromal cells

Due to the extensive variability between patient derived NAF and CAF, to complete Aim 1A we have decided to employ the use of murine-derived fibroblasts specifically for the seahorse experiments. We have shown that murine fibroblasts do express a CAF-like genetic signature with prolonged exposure to enzalutamide, therefore we have done experiments between naïve fibroblasts and those with a consistent exposure to enzalutamide. In addition, we will employ the use of CRISPR Cas9 technology solely on murine derived fibroblasts and not NAF and CAF. Moreover, we have seen advances in CRISPR Cas9 technology since the submission of this proposal. Hence, we changed our system from a double plasmid method to a single plasmid method (Lentiviral CRISPRV2 plasmid) which reduces cell selection time and has higher success rate (10).

Major Goal 3: To define the role of BHB in CRPC

Subtask 1: Generate OXCT1KO PCa cells
Subtask 2: Tissue recombination experiments

The Covid-19 pandemic caused a delay for in vivo experiments and hands-on work. The generation of OXCT1 epithelial knock outs on 22RV1 was significantly affected, however the project continues as planned. In addition, for the experiments involving co-culture, we have decided to employ the use of boyden chambers instead of the collagen mixture. Our rationale for such change relies on making our results specific to the activity of small molecules instead of cell interactions. Additionally, this method reduces the amount of cell death resulting from the collagen digestion and cell sorting procedure.

Products

Presentations:

Poster Presentation
Role of beta hydroxybutyrate in androgen receptor signaling inhibition therapy resistance.
SBUR 2018 Annual Meeting
Precision Medicine in Urology: Molecular Mechanisms, Diagnostics and Therapeutic Targets
November 8-11, 2018
Rancho Mirage, CA

Participants & Other Collaborating Organizations

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Months Worked:	12
Contribution:	Performing experiments, data analysis, data report.

Name:	Ashley Heard
Project Role:	Laboratory technician
ORCID ID:	Not registered
Months Worked:	7
Contribution:	Technical assistance with experiments.

Special Reporting Requirements
Nothing to report

Appendices Nothing to report

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