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The Opposing Roles of Oxidative Stress in Two Different Human Health Conditions

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ABSTRACT OF THE DISSERTATION

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Molecular Oxygen is one of the most important molecules in living organisms. Oxygen is the final electron acceptor in the Electron Transport Chain (ETC), which allows organisms to produce ATP. In mammals, oxygen and its chemically similar relatives, reactive oxygen species (ROS) can be used in signaling. For example, one ROS species, H_2O_2, can signal for apoptosis at low levels. In cancer, increasing the chance that a cell will undergo apoptosis can be useful. New drug targets are necessary for Castration Resistant Prostate Cancer (CRPC). Catalase, an enzyme that detoxifies the cell from H_2O_2, was shown to be a viable drug target for CRPC by demonstrating tumor reduction in mice. Catalase deficient cells also migrated slower, proliferated slower, and had altered adhesion.

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On the other hand, too much ROS in mammals can lead to DNA damage, as well as the oxidation of lipids and proteins. An accumulation of damaged DNA, lipids and proteins can lead to long term health impacts. ROS can be produced endogenously or can come from environmental sources like tobacco smoke. A historically underappreciated source of ROS is Third hand Smoke (THS). THS refers to the class of chemicals that reside in or on surfaces after secondhand tobacco smoke has settled and cleared. In this study, THS exposure was shown to increase the relative methylation in mitochondrial DNA. (mtDNA). This could possibly explain the metabolic phenotypes that has been observed in THS exposed mice in previous studies.

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Part I: Oxidative Stress and Prostate Cancer

In Aerobic organisms, the electron transport chain (ETC) allows for a controlled conversion of molecular oxygen to water through reduction creating a high proton concentration in the intermembrane space which generates the proton motive force to produce ATP, the energy currency of the cell. During this process, “electron leak” can occur leading to the production of the superoxide ion, O_2^-. This is one type of reactive oxygen species (ROS) [1]. To protect the cell from oxidative damage, there is a protein present in the mitochondria called Mitochondrial superoxide dismutase (MnSOD or SOD2). MnSOD will convert O_2^- to a slightly less reactive oxidant, hydrogen peroxide (H_2O_2)[2]. Other forms of ROS in the cell include hydrogen peroxide, singlet oxygen, or hydroxyl radicals. Although electron leakage is an endogenous source of ROS, there are many environmental sources of ROS that humans encounter. For example, polyaromatic hydrocarbons (PAHs) are formed from incomplete combustion of carbon-based fuel or cigarettes. Organisms have developed several mechanisms to detoxify the cell from different types of ROS. Superoxide dismutase 1 works on O_2^- in the cytoplasm,[3] while SOD3 works on the extracellular matrices (ECMs) of tissues.[4] Catalase (Cat) and glutathione peroxidase (GPx) both detoxify the cell from H_2O_2. Although H_2O_2 alone is not as reactive as other ROS species, in the presence of iron, H_2O_2 can generate oxidative damage via a Fenton reaction.[5] It is well established that excessive ROS can lead to DNA damage,

and contribute to cancer progression.[6] In addition, cellular conditions where ROS are too low are not ideal either. Abnormally low ROS may have implications in cancer because the lack of H_2O_2 prevents signaling for apoptosis.[7] Very low ROS in the cell also has the potential to perturb immune cell function, because ROS has a role in innate immunity.[8] In small concentrations, ROS also can act as a signaling molecule to trigger apoptosis or differentiation.[9] This is summarized in an illustration **(Figure 1)**. It is important to have an understanding of the role that ROS plays in signaling because high ROS and dysregulation of antioxidant response is known to be a key player in Prostate Cancer progression.[10,11]

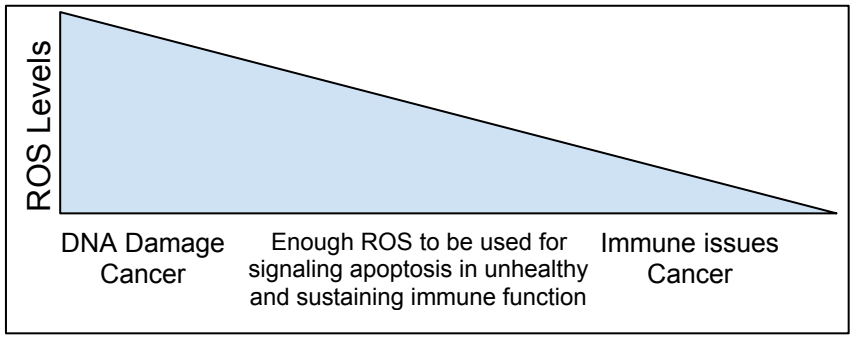

Figure 1. Summary of the impacts due to high or low ROS levels

Prostate Cancer (PCa) in America, is the second leading cause of cancer related death amongst people who have a prostate .[12] Although there has been a long-term decline in PCa deaths since the 1990s due to improvements to detection protocol, this decline stopped in 2013 and mortality rates have not improved since.[13] This indicates a need for novel drug targets and treatments. When caught early, prostate cancer tends to start as androgen dependent. At this stage, the PCa cells still rely on Androgen molecules to stimulate growth,

proliferation, and metastasis. While inactive, the Androgen receptor (AR) is in the cytoplasm complexed with heat shock protein 90 (HSP90). When it is met with an androgen molecule, either testosterone or dihydro-testosterone (DHT), the AR dissociates with HSP90, translocates into the cytoplasm, and begins to transcribe protoncogenes. Standard medical treatment for PCa consists of reducing androgens in the body through surgical castration or Androgen Deprivation Therapy (ADT). This leads to reduced transcriptional activity of the AR. ADT in some form has been around since the 1970s as demonstrated by a paper describing that PCa can be slowed by either taking estrogen or undergoing surgical castration[14]. Although surgical castration can still be used, we now have more sophisticated chemical castration drugs which have higher success in treating ADPC than estrogen did, especially if the disease is still localized.

There are three major types of ADT drugs, and they are each used in different stages of the disease. The first class of ADT drugs produced for PCa were luteinizing hormone-releasing hormone (LHRH) agonists. These drugs stopped androgen production at the pituitary level and are used to treat ADPC. They are usually the first treatment given, and unfortunately fail to work once the disease has reach castration resistance.

Castration Resistance isn't entirely understood but it has been well established that oxidative stress (OS) is the main driver for the development of CRPC's aggressive phenotype[18]. Unfortunately, most ADT has been shown to stimulate even higher OS in the body[19–21]. Therefore, the treatment that nearly

every PCa patient receives, elevates their risk for CRPC. Once the disease has progressed to CRPC or even worse, metastatic CRPC (mCRPC), it becomes extremely difficult to treat. When PCa metastasizes, it tends to settle onto thick bone (such as hip or thigh). Bone metastasis produces some of the worst symptoms across all cancer events. As the cancer grows the bones become weaker causing pain and fractures which can lead to secondary complications. In fact, in 70% of PCa related deaths, the autopsy will show metastasis to a bone site[22]. Prevention of mCRPC at the localized CRPC stage is necessary to prevent the most detrimental side effects of mCRPC.

First line of treatment against CRPC is to treat it with a class of drugs that work on many different cancers, taxanes. Taxanes are a class of chemotherapeutic drugs that target cells by binding to microtubules, stabilizing them, and preventing depolymerization. This leads to pushing rapidly dividing cells towards apoptosis,[23] which is useful for selectively killing cancer. Docetaxel is the specific type of taxanes used in CRPC. However, docetaxel resistance is common and can lead to the patient developing mCRPC while they are getting treatment for localized CRPC. In one cell culture study, it has been shown that Docetaxel treatment is associated with overexpression of p53 and p21 in a dose dependent manner. Cells with the high p53 expression exhibited less apoptosis from docetaxel and siRNA knockdown of P53 caused resensitization of the cancer, to the chemotherapy drugs.[24] This is in contrast to how p53 acts in relation to taxane resistance in several other cancers.[25] Stat1[26] and PIM1

Kinase[27] have also been found to have critical roles in the development of Docetaxel resistance. It has also been found that the NF-kappaB pathway plays a critical role in Taxane resistance in prostate cancer. In fact, when Docetaxel resistant cells were treated with an NF-kappaB inhibitor and Doxetaxel, more apoptosis was observed than Docetaxel treatment alone, indicating NF-kappaB expression contributed to Docetaxel resistance.[28]

Another class of drugs are called “First generation Anti-androgens” (FGAA). This includes drugs such as Flutamide, Bicalutamide, and Nilutamide which competitively bind to the AR and prevent activation[15]. They are typically used when the previous drugs no longer work. FGAA drugs are competitive inhibitors of the AR. By outcompeting Androgens for their binding spot on the AR, this drug class prevents translocation of the receptor to the nucleus, which prevents the transcription of proto-oncogenes and proliferation of the cancerous cells. This includes drugs like bicalutamide, nilutamide, or flutamide. Eventually however, most patients will fail to respond to FGAA and indicators of cancer progression like the Prostate-Specific Antigen (PSA) test will begin to show signs of cancer proliferation.

It has been observed that resistance to these drugs correlates with frequencies in point mutations in the AR suggesting a potential mechanism of resistance. Because of the nature of competitive inhibition, an increase in the concentration of ligand (DHT or Testosterone) can lead to a drug becoming less effective at the previously established therapeutic dose and due to side effects;

doses often cannot be increased. Another form of resistance to FGAA is the expression of splice variants of the AR that are constitutively active. Three major splice variants that lead to the truncated form of AR are: AR-V3, 7, and 9. These AR spice variants do not have their full ligand binding domain that typically interacts with Androgens, but they do contain the DNA binding domain. This leads to these transcriptions' factors being constitutively on.[29]

Second generation anti-androgens (SGAA) such as enzalutamide, apalutamide and darolutamide have similar capabilities to the FGAA, except that *in addition* they block the AR from binding to androgens. They also inhibit AR-DNA binding as well as AR translocation into the nucleus[16]. These drugs are sometimes used *instead* of FGAA and are sometimes used *after FGAA,* or Taxane-based treatments fail. Unfortunately, patients who receive ADT often can expect to develop Castration Resistant Prostate Cancer (CRPC) on average 2-3 years after initial ADT, which has a median survival rate range of 9-30 months [17].

SGAAs are one of the few drug classes that can overcome these specific resistance mechanisms, due to their ability to block AR-DNA binding. SGAAs include AR signaling inhibitors such as enzalutamide (Xtandi, ENZA, MDV-3100) and CYP17A1 inhibitors such as abiraterone acetate (Zytiga)[30]. Similar to FGAA, enzalutamide blocks Androgen-AR interaction but it has a higher binding affinity than previous drugs. SGAA have also been shown to inhibit coactivator recruitment, ligand-AR binding, and AR translocation to the nucleus[16]. This multi-target approach is why enzalutamide is more useful for dealing with AR splice

variants without ligand binding domains. Although this drug has worked to slow cancer growth in many patients, 25% of patients in the clinical study showed signs of primary resistance[31]. Although Abiraterone acetate was developed around the same time as enzalutamide, it works upstream of the AR. Rather than interacting directly with the AR or ligands, Abiraterone acetate prevents production of testosterone by inhibiting a key enzyme involved in androgen synthesis, Cytochrome P450 17A1 (CYP17A1). In an experiment where human CRPC was xenografted onto a mouse and treated with Abiraterone Acetate, an unexpected effect that it was observed was an increase in transcription of full length AR [32], and relatively less constitutively active, truncated AR. Although resistance to Abiraterone acetate has been observed in clinical settings, there are not many strong predictive factors of resistance in patients yet. Some potential predictive factors include serum PSA, AR mRNA sequencing for splice variants, and serum hemoglobin (Hb) concentration.[33] However, there is little to be done once a patient's cancer begins to proliferate despite the drugs.

Immunotherapy for PCa is available, however, the sipuleucel-T (provenge) vaccine only extends survival rates for about 4 months[34] on average. At the point in treatment where drug resistance is formed and the vaccine was given, there are no additional tools for preventing the cancer from metastasizing. Due to all these mechanisms of resistance, there is a need to find novel drug targets that can be used when both the vaccine and SGAA inevitably fails. Targeting the AR pathway has historically led to uncovering more androgen independent

phenotypes of PCa. Because of this, and the knowledge that ROS is an important player in CRPC progression, it could be more fruitful to pursue antioxidant targets. Due to the proliferation speed of cancer cells, they tend to accumulate more OS compared to its healthy tissue counterpart. High expression of antioxidant genes is sometimes observed to combat this, which can have an impact on drug resistance.

Taking advantage of OS to kill cancer is not a particularly new concept; many chemotherapeutic agents work by causing faster dividing cancer cells to generate enough OS to trigger apoptosis[35]. Associations between high catalase expression and multidrug resistance have been observed in different cancers[36,37]. Several antioxidant enzymes have been discovered to be involved in resistance to radiation therapy [38]. Catalase suppression has been shown to be effective at killing cancer cells in the past[39]. One study found that Catalase acts to protect cancer cells from ROS mediated apoptosis[40]. A follow up study from the same group found two compounds that can directly and indirectly suppress catalase and encourage apoptosis of cancer cells in vitro[41]. A natural product known to suppress cancer growth, wogonin, has been shown to promote apoptosis via suppression of catalase activity in cervical cancer cells, ovarian cancer cells, and non-small-cell lung cancer cells in vitro[42].

Although catalase suppression has found in vitro success, there is a need for more studies done in a three-dimensional tumor model like a tumor xenograft. A xenograft is when a foreign cell line (such as humans cancer cells) are grafted

into another organism. In this case, grafting human cancer cells into an immunodeficient mouse. Using a human derived cell line to create a tumor xenograft in a mouse is useful for several reasons. It uses human cells which are more relevant to human cancer than mouse tumors. It relies on a relatively inexpensive animal that reproduces quickly, allows for three-dimensional tumor growth, and the cells in culture can be manipulated for different experimental conditions before xenografting them into the mice. For example, a CRISPR-Cas9 enzyme can be used to edit, add, or inactivate a gene in the cancer cell line prior to xenografting. To test how catalase functions as a potential drug target in solid cancers, we decided to use the xenograft model. Rather than use compounds that have been known to inhibit catalase (and potentially have off target effects), we used a CRISPR related system, followed by a rescue to ensure that the changes we saw in tumor progression were due to tumor progression rather than off target effects. The findings were consistent with the prior studies that catalase suppression has the potential to slow the growth of even one of the most drug resistant PCa cell lines.

Part 2: Third Hand Smoke and Metabolism

As discussed above, high exposure to ROS is not ideal either. In the case of cancer, internally generated ROS is the focus, but there are also external sources of ROS. External forms of ROS can include: radiation, pollution, industrial solvents, pesticides, some drugs, and cigarette smoke.[43] It has been well established that exposure to cigarette smoke causes an increase in oxidative stress in the body.[44,45] It is well known that cigarette smoke poses a health risk to both the smoker, who inhales first hand smoke (FHS) and second hand smoke (SHS), and nearby nonsmokers who inhale SHS. It is less commonly known however, that SHS doesn't entirely "clear" from a space it has contaminated. In fact, as summarized in **Figure 2,** many of the health impacting compounds that exist in cigarette smoke can accumulate on surfaces; this is what we now know as **Third Hand Smoke (THS)**. Some of the first studies of THS included one in 1991 that analyzed dust in smokers' homes and found that it

Figure 2. How Third Hand Smoke (THS) accumulates

was contaminated with high levels of nicotine[46]. Another study published in 2004 compared nonsmokers, outdoor smokers, and indoor smokers and found that the homes of outdoor smokers contained nicotine dust levels that were 5-7 times higher than those of nonsmokers[47]. Indoor smoking households reported 3-8 times the nicotine levels then outdoor smoking households. Studies done in the cars of smoker's vs nonsmokers showed similar results[48].

Although the above studies done to characterize the level of nicotine were key to discovering that accumulation of environmental tobacco residue is a potential problem, they did not provide any direct evidence that THS is causing any impacts on human health. Further studies had to be done to evaluate: 1) if THS associated compounds are found in high enough concentrations in smokers' homes to impact human health, and 2) if these compounds linger long enough to cause health impacts over time. When nicotine is burned in a cigarette it is also oxidized, which forms compounds called tobacco-specific nitrosamines (TSNs).[49] These compounds are very reactive and have been established as carcinogens [50]. TSNs can continue to react with oxygen, low levels of ozone, dust, or other air pollutants to create even longer lasting compounds. One study has shown that THS lingers even two months after smokers have moved out of a residence[51]. Cotinine is a metabolite produced by humans when nicotine is metabolized. It can be detected in urine as an indicator of nicotine exposure. It has been shown that when non-smokers move into a residence that previously housed smokers, the new, non-smoking residents show 2-7 times higher levels of cotinine in the

urine compared to residents who move into a former nonsmoking residence[51]. Because these studies demonstrate that THS lingers long, contains reactive compounds in high enough concentration to be detected in humans, it was likely that THS was impacting human health. Many of these studies however, relied on self-reporting of smoking habits which can be unreliable. To show with certainty that THS alone was contributing to the cotinine levels, controlled studies need to be conducted in an environment where there is no risk of SHS exposure. Since studies of some of these TSNs have already been done in cell culture, the next step is to look at THS in small mammals with similar biomarkers to humans. Mice are a good choice since they reproduce quickly and they can be tested for many of the same biomarkers we use in human studies, such as cotinine.

It is important to understand the health impacts of THS because it can lead to changes in attitude towards smoking[52] which could result in public policy changes that limit the exposure to the population. A study in 2016[53] showed that, in mice, THS exposure contributes to an increase of fat deposits in the liver, high insulin in the serum, high blood sugar, and decreased activity of some antioxidant enzymes. The authors describe this as being like metabolic syndrome. In addition, this study puts THS-exposed mice and clean air control mice on a “Western, High Fat" diet (HFD) or a standard diet (SD). Interestingly, the HF clean air group did not have statistically significant increases in fasting blood glucose, serum insulin, or body weight. This shows that the increase in fat intake alone was not sufficient to change these biomarkers. THS alone without a

high fat diet did cause differences in serum insulin and blood glucose levels, it did not have an impact on average body size by itself. The combination of western diet and THS exposure however, caused an increase in serum insulin, blood glucose, and body weight that was significantly higher than both the THS SD, and the clean air HFD. This evidence supports that THS exposure is potentially even more dangerous in regions where fat intake tends to be higher. Another study in 2018 that looked at THS exposure over time, found decreased ATP production in the liver as early as 2 months. All this evidence suggests that THS causes major problems in the metabolic health in mice. The mechanism underlying this metabolic shift is still yet to be investigated.

Tobacco use has already been established to cause genetic and epigenetic alterations in the case of FHS and SHS. Epigenetics is a term that encompasses any factors that impact gene expression, but do not directly involve changing the DNA's sequence directly. Some examples of epigenetic changes include modifications of histone proteins, DNA methylation, and ncRNAs. DNA methylation is a specific form of epigenetic regulation that involves a class of enzymes called DNA methyltransferases (Dnmts)[54,55]. Dnmts catalyze the covalent bonding of a methyl functional group to the 5th carbon in the cytosine base in DNA. Methylated cytosines are usually followed by guanine (CpG). There are three major methyltransferases: Dnmt1, Dnmt3a, Dnmt3b. Dnmt1 is known as the "maintenance" methyltransferase because it specifically methylates the new sister chromatids after DNA replication[56]. Dnmt3a and Dnmt3b however, are

known as the de novo methyltransferases because they can methylate DNA that has not been methylated previously. When an increase of CpG methylation is found on a gene, it often leads to a decrease in expression of that gene. For example, a study in 2012 found that participants exposed to SHS and air pollution were more likely to have hypermethylation *and* decreased expression of the genes IFN-γ and Foxp3, depending on the tissue type analyzed[57]. Several genome wide studies have found associations between tobacco use and an increase in DNA methylation[58–61].

Given that THS impacts ATP production[62] and fatty oxidation,[53] the mitochondria is likely being impacted. Mitochondria are the organelles responsible for ATP production and even encompasses its own **DNA (mtDNA)** that encoded for some of the proteins that are responsible for ATP production. Interestingly, there is a well characterized association between **nonalcoholic fatty liver disease (NAFLD)** and an increase of mtDNA methylation.[63,64] There are also several studies that show associations between NAFLD and methylation for nuclear encoded genes that regulate mitochondrial function.[65,66] Since THS has been shown in mice to have an increase in fatty deposits in the liver, it's possible that THS is working through a similar mechanism in the mitochondria. In fact, SHS exposure in children has been shown to be associated with development of NAFLD[67].

Tobacco exposure in its various forms have been shown to cause mitochondrial dysfunction. One of most known effects of smoking during

pregnancy, low birthweight, was found to occur due to mitochondrial dysfunction.[68] Similarly, it was found that in the skeletal muscle of COPD patients, mitochondrial dysfunction due to cigarette smoke is not only critical to progression of the disease, but it is highlighted as a potential place for intervention.[69] Even more modern forms of tobacco use, such as tobacco e-cigarettes, have been found to cause mitochondrial dysfunction in lung epithelial cells.[70]

Considering the impact FHS and SHS have on mitochondria, it is not entirely surprising to see that THS can influence metabolism as well. One study even found an association between tobacco use in pregnant people and methylation of mitochondrial DNA (mtDNA) in the placental tissue.[54] An increase in mtDNA methylation that has been linked to mitochondrial dysfunction has also been found in those exposed to traffic related air pollution[71]. This combined with the known effects of THS indicates that THS is likely causing a decrease in ATP production via mitochondrial dysfunction. Given the literature showing that other forms of tobacco exposure led to epigenetic alterations and DNA mutations, we decided to focus on the impact THS has on mitochondrial DNA.

The THS exposure system used was the same as what has been described previously.[72,73] Briefly, a Teague smoking system was used to expose different household fabric to cigarette smoke. The fabric is then removed from the smoke exposure chamber and placed in the cages of C57/bl6 mice. After 4 months of exposure, the mice were euthanized, and the livers were studied. We

found that THS leads to an increase in mtDNA methylation and mtDNA mutations**.** Unsurprisingly, these findings mean that much like FHS and SHS, THS can cause mtDNA damage, which may contribute to the metabolic impacts we see in THS exposed mice.

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Chapter 2: Catalase as a Novel Drug Target for Metastatic Castration-Resistant Prostate Cancer

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ABSTRACT

Prostate Cancer (PCa) is the second most prevalent cancer in the world. Currently, most treatments for PCa involve Androgen Deprivation Therapy (ADT) which inhibits androgen-dependent tumor cell growth. When PCa is diagnosed early and is still Androgen Dependent, ADT is effective. However, this therapy is not effective for metastatic Castration-Resistant Prostate Cancer (mCRPC). Although the mechanism of becoming Castration-Resistant is not fully understood, it is known that high levels of oxidative stress (OS) are important for cancer suppression. Catalase is a very important enzyme in controlling OS levels. We hypothesized that catalase's function is critical for the progression to mCRPC. To test this hypothesis we used a CRISPR nickase system to create a catalase knockdown in PC3 cells, a mCRPC human-derived cell line. We obtained a $Cat^{+/-}$ knockdown cell line, which has approximately half of the transcripts for catalase, half of the protein levels, and half of catalase activity. The $Cat^{+/-}$ cells are also about twice as sensitive to H_2O_2 exposure compared to WT cells, migrate poorly, have low attachment to collagen, high attachment to Matrigel, and proliferate slowly. Using SCID mice for a xenograft model, we show that $Cat^{+/-}$ cells form smaller tumors than wild-type tumors with less collagen and no blood vessels. These results were validated via rescue experiments where functional catalase was reintroduced into the $Cat^{+/-}$ cells and the phenotypes were reversed. This study shows a novel role for catalase in deterring mCRPC development and points to a new potential drug target for mCRPC progression.

Summary

Novel treatments for Metastatic Castration-Resistant Prostate Cancer are needed. By taking advantage of the sensitivity of tumor cells to oxidative stress (OS), reducing an enzyme, catalase, that decreases OS, has the potential to provide another target for Prostate Cancer therapy.

INTRODUCTION

Prostate cancer (PCa) is the second leading cause of cancer-related deaths in American men, with 1 in 9 expected to be diagnosed in their lifetime [https://www.cancer.org/cancer/prostate-cancer/about/key-statistics.html]. Currently, most treatment strategies include Androgen Deprivation Therapy (ADT) which can either involve blocking the production of androgens, blocking the androgen receptor (AR) with agonists, or preventing the translocation of the AR from the cytoplasm to the nucleus where it acts as a transcription factor for pro-oncogenic genes [1]. However, this strategy is only effective in cases of Androgen-Dependent Prostate Cancer (ADPC). ADPC that has not metastasized typically has a positive response to this therapy, but in most cases, PCa will progress to a metastatic Castration-Resistant Phenotype (mCRPC) in 2-3 years [1]. CRPC can grow without the presence of androgens, and also tends to respond poorly to most other treatments, including more general proliferation-targeting cancer drugs such as docetaxel [2] because PCa cells divide slowly. Once CRPC metastasizes, the median survival is three years [3].

Discussions regarding the shift in aggression from ADPC to mCRPC have been focused on the upregulation of AR that acts as a transcription factor and turns on genes involved in PCa cell proliferation [4-6]. However, it has been shown that only about 20% of mCRPC have an increase of AR compared to ADPC [7]. In mCRPC, ADT drugs such as Leuprolide, that function to lower overall testosterone, fail because of the intracrine androgen synthesis by PCa

cells [8,9]. While Cytochrome P450 inhibiting drugs, such as abiraterone acetate, have shown promise to extend mADPC survival by several months [10], this drug functions to block Androgen production and will not be effective for PCa that is entirely Hormone-Refractory. For mCRPC, an immunotherapy is available, sipuleucel-T, but is costly and only increases survival by about 4.5 months [11]. These findings point to the need for novel drug targets for the treatment of mCRPC.

Although the mechanism of Castration-Resistance is not entirely understood, it is known that oxidative stress is necessary for this aggressive phenotype to develop [7,12,13]. Tumor cells that are castration-resistant need a mechanism of evading cell death while there are overwhelming levels of Reactive Oxygen Species (ROS) in their microenvironment, generated from their persistent proliferative state [14]. The high levels of ROS create a microenvironment that could be used for new drug therapies because tumor cells will be more susceptible to ROS-mediated apoptosis than the cells in healthy tissues.

It has been observed that antioxidant enzymes are typically dysregulated in cancer [12,13,15-20]. For example, overexpression of catalase, the antioxidant enzyme required for detoxification of H_2O_2, has been observed in several cancers, [21-24] including PCa [18]. It has been reported that in a mouse sarcoma cell line, catalase “protects” tumor cells from H_2O_2 induced apoptosis [20]. Catalase suppression, either through natural products or the

potent catalase inhibitor, 3-amino-1:2:4:-triazole (ATZ), has been shown to trigger apoptosis in various cancer cell lines including ovarian, breast, and gastric [16,25], Given that targeting the AR receptor pathway has become an arms race against the rise of different mechanisms of resistance, we sought to take advantage of the increased oxidative stress in the tumor microenvironment by targeting catalase.

Therefore, *we hypothesized* that knocking down catalase in PC-3 cells, a CRPC cell line isolated from a human metastatic lesion in the bone, will reduce cell proliferation and tumor progression. Our study presents strong evidence that catalase reduces cell migration, proliferation, and tumor development making it a viable drug target opportunity for PCa management.

MATERIALS AND METHODS

Cell Culture. The PC-3 cell line is an androgen-independent prostate cancer cell line that was purchased from American Type Cell Culture Collection (Manassas, VA). Cat +/- is a PC-3 cell line obtained in our laboratory that has a disrupted Catalase gene in one allele leading to 50% Catalase expression/production/activity. Both cell lines were cultured on 0.1% Collagen Coated Tissue Culture plates at 37ºC with 5% CO_2 and in RPMI-1640 (Sigma R8758) supplemented with 10% FBS, and 1% Penicillin (100IU/ml)-Streptomycin (100µg/ml). Media renewal was done as needed about 2-3 times per week. Unless otherwise specified, cells were prepared to be assayed by seeding at 3.0 x 10^4 cells/cm^2 and grown to about 80% confluency.

CRISPR System. CRISPR Double Nickase expression plasmid and sgRNA expressing plasmid were purchased from Santa Cruz Biotech (sc-400353-NIC). The Nickase was chosen to reduce the probability of off-target effects. Cells were transfected with 2µg of plasmid DNA using Lipofectamine 2000 Transfection Reagent (Thermofisher 11668027). After 24 hr cells that were successfully transfected were selected using puromycin treatment at 10µg/ml. Selection media was maintained for 48 hours. Surviving cells were then plated in a 96-well plate at a density of 0.3 cells/well to ensure each well contained a colony derived from a single cell. The various colonies were cultured under normal conditions until they were screened via RT-qPCR or immunoblot. The following gRNA sequences were used in this kit: Plus Strand, Gttattacagtagggccccg, and Minus Strand: ggttaccagctccagtggt to lead the CRISPR-NIC enzyme to an appropriate region on the Catalase gene.

Immunoblot analysis. The cells were washed with PBS then lysed in Radioimmunoprecipitation assay buffer (RIPA: 10mM NaCl, 50mM Tris pH 7.5, 5mM of EDTA, 1% NP40, 1% Sodium Deoxycholate, 0.1% SDSm pH7.5) and centrifuged for 15 min at 10,000 x g to remove cell debris. The supernatants were removed and aliquoted for subsequent use. Protein concentrations were determined using the DC Protein Assay kit (Bio Rad). Samples were prepared to run on the gels by adding a loading buffer containing sodium dodecyl sulfate (SDS) and heating at 95^{o}C for 3 min. Proteins were separated by size via 12% SDS-Polyacrylamide gel electrophoresis (PAGE) followed by an overnight 45V

wet tank transfer system (Bio-Rad) onto a nitrocellulose membrane. Primary antibodies consisted of anti-catalase antibodies (Abcam ab16731) diluted 1:1000 and antibodies to the reference gene, Histone 3 (cell signaling cat# 9717) diluted 1:1000. 2º Antibodies: Ms-Gt HRP (1:500); Rb-Gt HRP (1:2000) were purchased from ABCAM ab97023 and ab970, respectively.

Total RNA Extraction. Cells lines were washed in PBS and directly lysed on the plate with Trizol Reagent (Thermofischer 15596026). RNA was purified from the Trizol solution by the manufacturer's protocol using Direct- zol Kit (Zymo R2051). Briefly, lysates were centrifuged at 12,000 x g for 5 min to remove cell debris. The supernatant was loaded onto a spin column and centrifuged for 1min at 12,000 x g. Flow-through was discarded. A DNAse treatment was done using the DNAse I in DNA digestion buffer supplied in the kit. RNA wash buffer was loaded onto the column to remove remaining Trizol and wash the RNA. The wash step was repeated and RNA was eluded in 30µl of DNAse/Rnase-Free water.

Real-Time qPCR. cDNA was synthesized using 500 ng of RNA with the PrimeScript RT reagent Kit (Takara RR037A) according to the manufacturer's protocol. qPCR results were normalized to β-actin as a reference gene. 2µl of cDNA templet were mixed with a 23µl master mix containing 12.5µl SYBR Green (Bio-Rad), and 150nM of oligonucleotide primers. PCR was carried out in a Bio-Rad Biorad CFX Connect real-time PCR detection system (Bio-Rad, Hercules, CA). The thermal profile was 95 ºC for 2min followed by 40 amplification cycles,

consisting of denaturation at 95 ºC for 15s, annealing at 60 ºC for 60s. Fluorescence was recorded after every cycle for quantitation purposes. A melt curve analysis was used to verify a single product consisting of heating from 65ºC-95 with increments of 0.5ºC for 5s. The following oligonucleotide sequences were designed using IDT primer quest and verified with NCBI Primer-Blast. Beta-actin *Forward* GCTAAGTCCTGCCCTCATTT *Reverse* GTACAGGTCTTTGCGGATGT Catalase: *Forward* CTGGGAGACGAGACACATAAAC *Reverse* TGGTCACTCCCTCTACATTCT

Catalase Activity. Catalase activity was determined in nmol/min/ml using a Catalase Activity kit (Cayman Chemicals 707002). Briefly, cells were washed twice with PBS, scraped, counted, and centrifuged for 5min at 125 x g. The cells were then lysed in cold buffer (50mM potassium phosphate, pH 7.0, containing 1mM EDTA). According to manufacturer's protocol, activity was measured by incubating cell extract containing catalase with methanol which will produce formaldehyde in the presence of an optimal concentration of H_2O_2. Formaldehyde production can be measured by adding 4-amino-3-hydeazino-5-mercapto-1,2,4,triazole (Purpald) which changes from colorless to purple in the presence of aldehydes. The change in color was quantified using a Plate reader (BioTek, Winooski, VT) at 540nm.

Cell Proliferation. Cells were seeded in six-well plates at a cell density of 3.0 x 10^4 cells/cm^2. Each day, cells were washed with sterile 1x PBS followed by trypsinization and counted using a hemocytometer.

H_2O_2 Toxicity. Cells were seeded in six-well plates at a cell density of 3.0 x 10^4 cells/cm^2. One day post-seeding concentrations of 0μM, 25μM, 50μM, and 100μM were added to cells. H_2O_2 was diluted in sterile PBS, immediately before use, and added also with each media change. Each day, cells were washed with sterile 1x PBS followed by trypsinization and counting to determine the effects of H_2O_2 on the growth rate of the cells.

Adhesion Assay. PC-3 cells were plated on a collagen-coated 6-well plate at a density of 3.0 x 10^4 cells/cm^2 and allowed to grow to about 80% confluency. Media was aspirated and cells were washed with PBS. A pre-warmed 0.25% Trypsin EDTA solution was added and the cells were allowed to detach at room temperature and under observation in the microscope. The required time needed for all the cells to detach was used to quantify cell adhesiveness.

Migration Assay. Confluent (95-100%) WT PC3 cells and Cat+/- PC-3 cells, were wounded with a sterile pipette tip by making a scratch across the center of the plate. Cell migration was determined by measuring the distance between the wounded edge to the leading edge of migration at time points 0, 12, 24, 48 hr, post-wounding.

Xenograft. Experiments were approved by the Institutional Animal Care and Use Committee of the University of California, Riverside. Male immunodeficient SCID Mice (4-5 weeks old) were anesthetized and injected subcutaneously in the area of the prostate with 10^7 of WT or Cat+/- PC-3 cells in 100μl of PBS. They were allowed to grow for 7 weeks. At 7 weeks, mice were euthanized and tumors

were photographed, weighed, volume was measured, and then prepared for histology.

Histology. Steps are performed at room temperature unless otherwise indicated. Each tumor was cut in half and incubated in 4% Paraformaldehyde overnight at 4ºC on a rocker. Then the tissue was washed in PBS three times for 15 min with gentle shaking followed by incubation in 0.1M Glycine in PBS for 30 min with gentle shaking. This was followed by incubation in a 15% sucrose solution for 4 hr at 4ºC followed by overnight incubation in 30% sucrose at 4ºC. Tissues were rinsed with PBS and embedded into Optimal cutting temperature compound (OCT) and frozen using a dry ice ethanol slush. 8-10μm sections were prepared and then stained with Hematoxylin and Eosin or Mason Trichome as previously described [26].

Statistics. Differences between groups were assessed using a student's t-test in Graphpad Instat Software (Graphpad, La Jolla, CA, USA). Data is represented as mean +/- Standard Deviation represented as error bars. qPCR statistics was performed using CFX Maestro™ software (BioRad, Hercules, CA, USA) and displayed the data as ddCT +/- SEM as error bars.

RESULTS

PC-3 Cells are Dependent on Catalase for Growth

We evaluated proliferation of WT PC-3 cells while under inhibition of catalase using a specific inhibitor of this enzyme, 3-Amino-1,2,4-triazole (ATZ) (**Supplementary Fig. 1)**. PC-3 cells were treated with 250mM ATZ which is ½ of the LD50 of ATZ for this particular cell line. We found that over the span of 5 days, inhibition of catalase resulted in very low proliferation and survival. It is clear that after only 24 hours of growth with the inhibitor, PC3 cells do not grow well. This indicated that catalase is important for the growth of the PC3 cells and could become a potential target to slow tumor growth in Castration-Resistant Prostate Cancer Tumors.

Catalase Knockdown Results in Altered Cellular Processes

Using a CRISPR Double Nickase system, we produced a clonal population of PC-3 cells in which the catalase gene was disrupted in one allele of the chromosome ($Cat^{+/-}$); (**Fig 1A**). The WT cells are the original PC3 cells purchased from ATCC. Because our sequencing results showed a 4bp deletion, we anticipate a complete loss of functionality in this allele due to a frameshift mutation. As expected for a stable knockdown of $Cat^{+/-}$ we observed

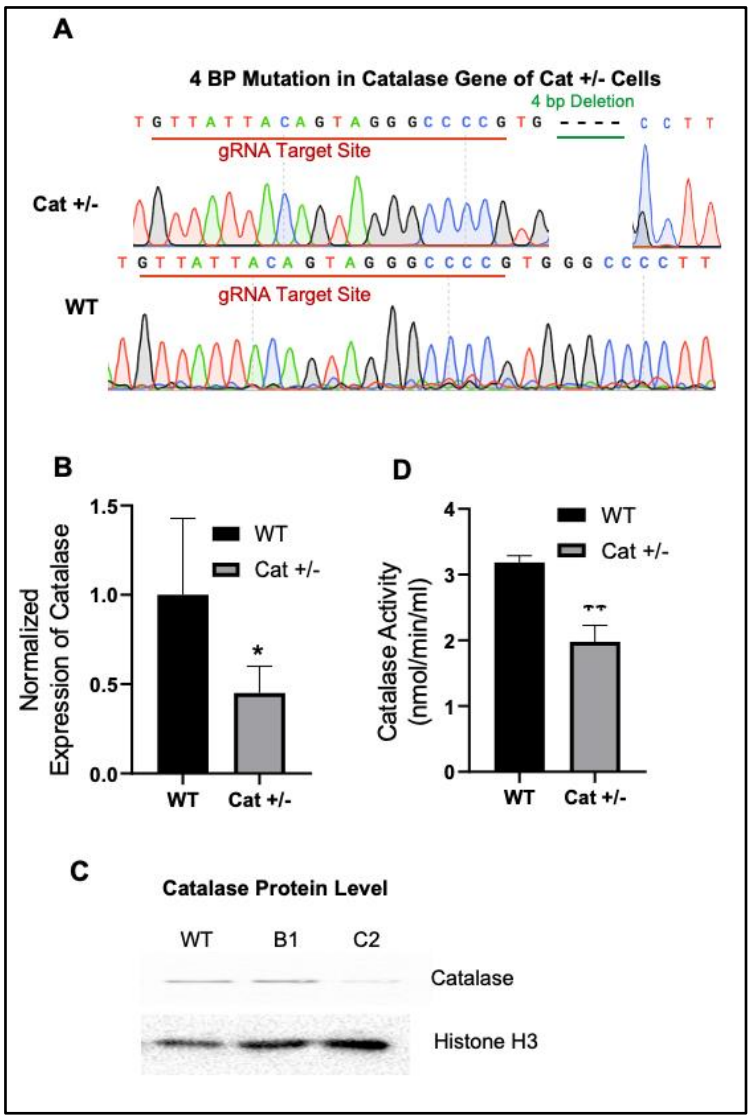

Figure 1. Catalase enzyme is successfully knocked down in PC-3 Cells. Verification of knockdown in a clonal PC-3 line done with various methods. (**A**) Sanger sequencing of CRISPR target area revealing a 4pb deletion. (**B**) RT-PCR results showing decreased catalase mRNA levels. (**C**) Western blot showing decreased catalase protein levels in two clonal populations after CRISPR. Clone "C2" was chosen for further screening of catalase activity loss. Clone "B1" did not have a lower catalase expression **(D**) Catalase activity assay showing decreased catalase activity for the $Cat^{+/-}$ cells. All data are Mean ± Standard Deviation *= p< 0.05, **=p<.01, n = 3, 35mm plates/time point.

approximately a 50% reduction in catalase mRNA transcript (**Fig. 1B**), protein levels (**Fig. 1C**), and enzymatic activity (**Fig. 1D**).

Given that high proliferation is a major hallmark of cancer, we determined whether the lower catalase expression and activity in the $Cat^{+/-}$ line leads to a decrease in cell proliferation. Using cell counting over a course of six days to determine proliferation levels, we found that $Cat^{+/-}$ cells show a lower number of cells as early as day two of growth, with significantly fewer cells on days 3, 5, and 6 (**Fig. 2A**).

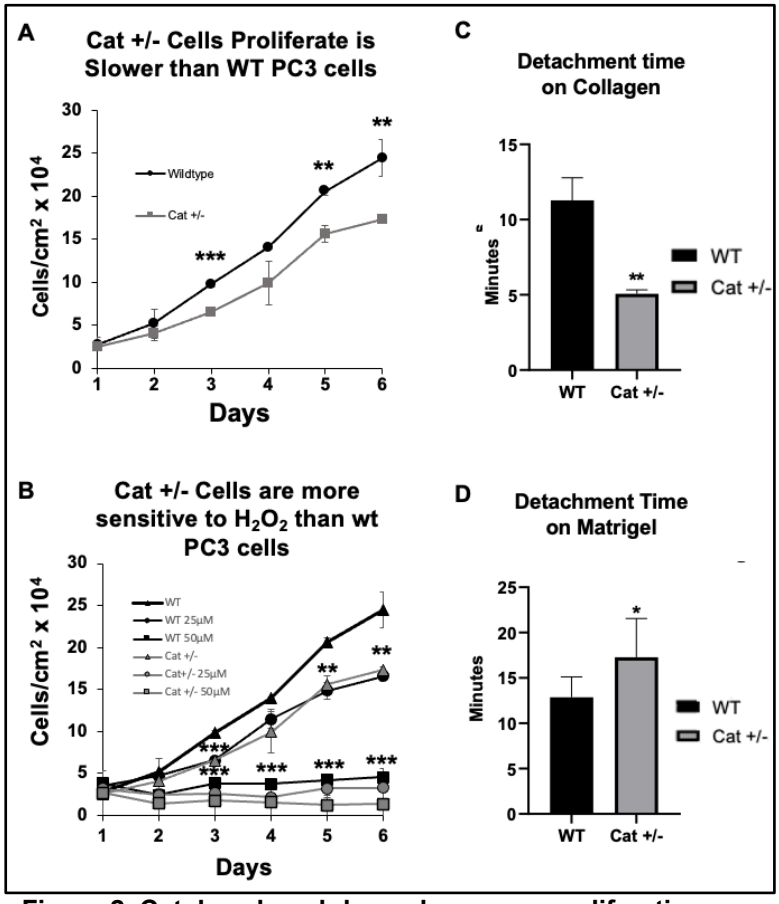

Figure 2. Catalase knockdown decreases proliferation, increases susceptibility to H_2O_2 toxicity, decreases adhesion on collagen, but increases adhesion on matrigel. (**A**) $Cat^{+/-}$ Cells proliferate slower compared to WT PC3 cells under standard growth conditions**.** **(B**) Cat +/- cells are about twice as sensitive to H_2O_2 when compared to WT PC-3 cells. (**C**) $Cat^{+/-}$ cells are less adhesive on Collagen then WTPC-3 cells. (**D**) $Cat^{+/-}$ Cells are more adhesive on Matrigel than WT PC-3 cells. All data are Mean ± Standard Deviation *= p< 0.05, **=p<.01, **=p<.001; n = 3, 35mm plates/time point.

In order to show that the catalase knockdown cells are deficient in response to oxidative stress induced by H_2O_2 exposure, the growth curve was repeated with varying concentrations of H_2O_2 (**Fig. 2B**). WT PC-3 cells exposed

to 25 μM of H_2O_2 proliferated comparably to $Cat^{+/-}$ cell with no exposure to H_2O_2. When $Cat^{+/-}$ cells were exposed to any concentration of H_2O_2, there was a decreasing number of cells each day. In contrast, it required 50μM of H_2O_2 treatment to induce similar levels of H_2O_2 toxicity in WT (**Fig. 2B**).

$Cat^{+/-}$ cells also show perturbed adhesion. When plated on collagen, WT PC3 cells require over twice as long to fully become detached from the plate, showing that catalase loss decreases cell adhesion on collagen (**Fig 2C)**. At room temperature, it requires about 11 min for WT to detach, whereas it takes about 5 min for $Cat^{+/-}$ cells to detach. In contrast, on Matrigel, $Cat^{+/-}$ cells show a slight, yet significant increase in adhesion compared to WT PC3 cells. At room temperature, PC3 cells detach from Matrigel in about 12 ½ min on average, whereas Cat +/- took about 17 min to detach (**Fig 2D)**.

Another characteristic of cancer cells is the ability to migrate and metastasize. Migration can be evaluated *in vitro* by the scratch assay[26]. We plated the cells on collagen, made a scratch when the cells were confluent, and then compared the migration into the scratched space by the $Cat^{+/-}$ and the WT PC-3 by measuring the distance from the edge of the scratch to the front of the migrating cells at 12, 24, and 48 hr. Overall, Cat +/- cells migrate more slowly than WT (**Fig. 3A, B).** By the end of 48 hr, WT cells had migrated to completely close the gap, whereas $Cat^{+/-}$ cells had only migrated to about 50% closure **(Fig. 3 A, B**).

When seeded in Matrigel, Cat$^{+/-}$ cells also show slower migration (**Fig. 3 C,D)**, although the difference between the WT and Cat$^{+/-}$ migration is not significant until 12 hr (**Fig. 3D**). By 24, and 48 hr the migration of the Cat+/- cells is hindered even more on Matrigel than on collagen with WT cells closing the gap on average by 80% and Cat$^{+/-}$ cells closing the gap on average only 10% at 48hr (**Fig. 3D)**.

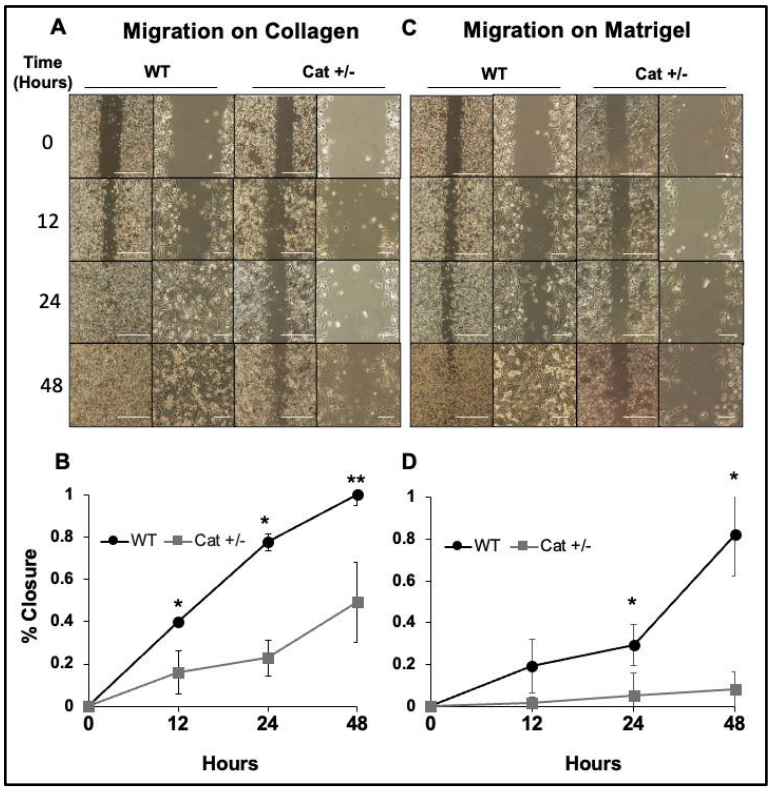

Figure 3. Catalase knockdown cells grown on collagen and matrigel have decreased migration in response to injury. Cat$^{+/-}$ cells and WT PC-3 cells were grown on 35mm to complete confluency and then wounded. (**A**) Slower percent closure over time was measured for Cat$^{+/-}$ Cells on Collagen. Representative photos of Cat +/- and WT PC-3 cells migrating on Collagen. (**B**) Quantitation of percent wound closure on collagen using image J. (**C**) Representative photos of Cat$^{+/-}$ and WT PC-3 cells migrating on Matrigel. (**D**) Quantitation of percent closure on matrigel using ImageJ. All data are Mean ± Standard Deviation *= p< 0.05, **=p<.01, n = 3, 35mm plates/time point. Scale bars are 500 and 100 nm for lower and higher magnification, respectively.

Gain of Catalase Function Reverses the Altered Cellular Processes

To ensure that these observations are a direct result of catalase loss and not an off-target effect of the CRISPR system, we performed rescue experiments by stably transfecting a catalase expressing plasmid into the Cat$^{+/-}$ cell line. Successful transfection of the plasmid was validated via PCR amplification of the

promoter region of the neomycin resistance cassette in transfected cells, but not in non-transfected cells (**Fig 4A)**. To show that the plasmid is in fact expressing functional catalase, we also determined that there is increased catalase activity levels even compared to PC-3 cells (**Fig. 4B)**. This indicates that we have rescued catalase activity (Rcat$^{+/-}$).

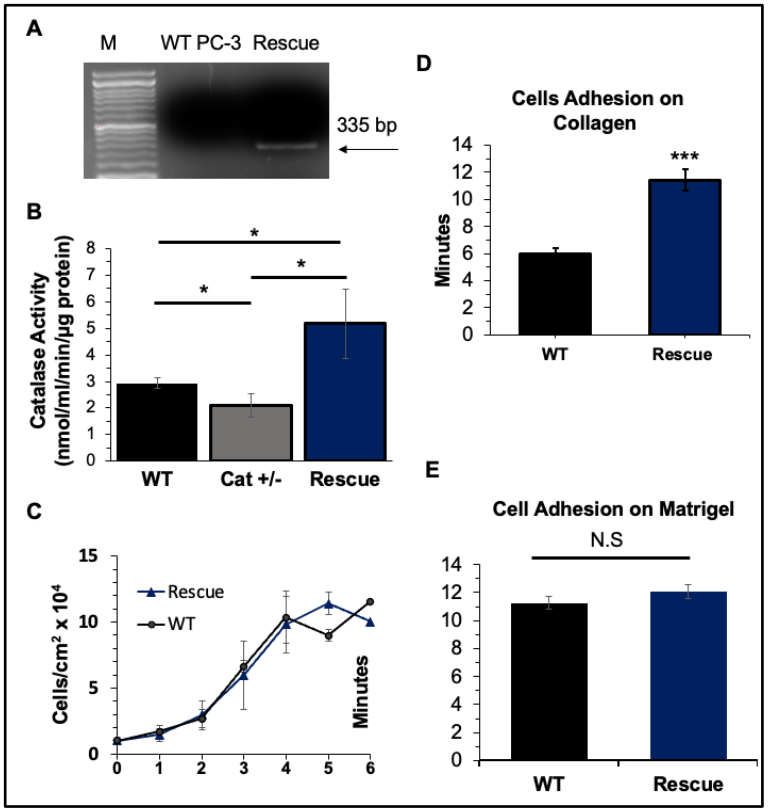

Figure 4. Catalase rescue was established in the knockdown background. (A) PCR of Neomycin resistance gene in rescue plasmid only amplifies this gene in RCat$^{+/-}$ DNA. **(B)** Catalase activity showing that RCat$^{+/-}$ has activity levels that equal to or exceed that in WT PC-3 cells. **(C)** Cat$^{+/-}$ cells and WT PC-3 cells were grown on 35mm dishes and proliferation was determined by counting. RCat$^{+/-}$ cells show a comparable rate of proliferation to WT PC-3. (**D)** RCat$^{+/-}$ cells show even higher adhesion to collagen than WT PC-3 cells do. **(E)** RCat$^{+/-}$ cells seeded on Matrigel have comparable adhesion to WT PC-3 cells. All data are Mean ± Standard Deviation *= p< 0.05, **=p<.01, **=p<.001; n=3, 35mm plates/time point

We found that when Catalase was reintroduced into the cells, proliferation increased to become similar to the proliferation of WT PC3 cells (**Fig. 4C).** Also, the alterations in adhesion we observed in the Cat$^{+/-}$ were reversed in the RCat$^{+/-}$ line. On collagen, the RCat$^{+/-}$ line showed a significant increase in adhesion compared to WT PC-3 cells **(Fig. 4D)** whereas on Matrigel, the RCat$^{+/-}$ was not significantly different from WT PC-3

cells (**Fig. 4E).** This latter result also shows that the plasmid used for transfection does not have secondary effects on the cells. In addition, we determined that RCat$^{+/-}$ migrated similarly to WT PC-3 cells **(Fig 5).** This occurs both on collagen (**Fig. 5A,B)** and Matrigel (**Fig. 5C,D).**

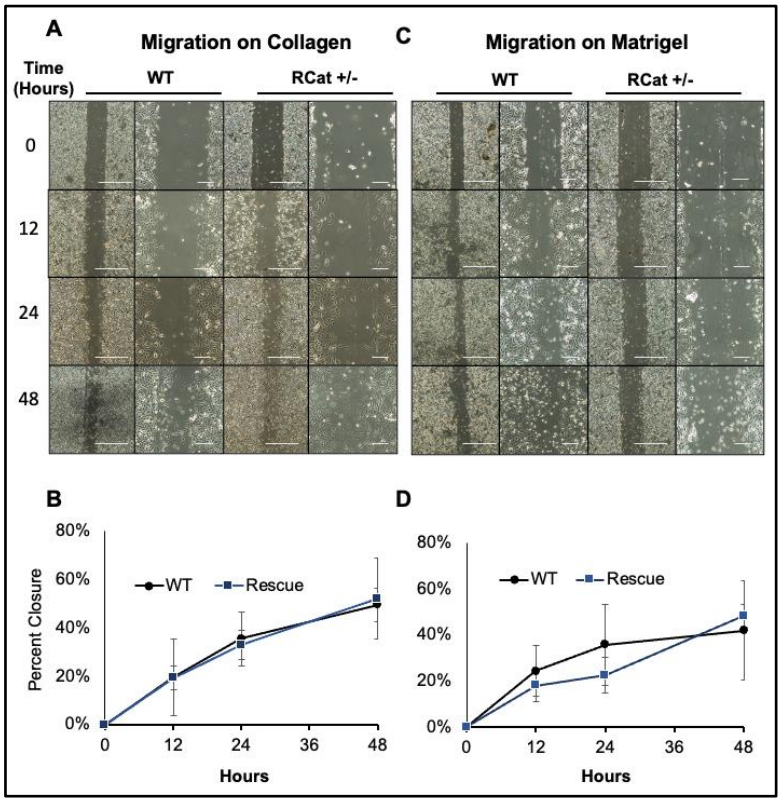

Figure 5. Catalase is responsible for decreased migration in response to injury. Percent closure was measured for RCat$^{+/-}$ cells cultures on (**A,B)** Collagen and (**C,D**) Matrigel. Representative photos of Rcat$^{+/-}$ Cells migrate in response to injury during a scratch assay slower than control WT cells on both (**A,B**) Collagen and (**C,D**) Matrigel. All data are Mean ± Standard Deviation, No significance; n = 3, 35mm plates/time point. Scale bars are 500 and 100 nm for 4x and 10x, respectively.

Catalase Knockdown Results in Smaller Tumor Size *In Vivo*

Although *in vitro* migration and proliferation assays can offer insight into tumor biology, to determine whether catalase plays a role in prostate tumor growth, we used a xenograft model to examine whether there were differences in tumor growth *in vivo*. WT and Cat$^{+/-}$ PC-3 cells were xenografted into SCID

immunodeficient mice. After 7 weeks of tumor growth, mice were euthanized, tumors were removed and then photographed showing that overall, Cat$^{+/-}$ cells formed significantly smaller tumors than WT PC3 cells (**Fig 6A)**. All WT tumors had blood vessels indicated by blue arrows, whereas the Cat$^{+/-}$ tumors did not (**Fig. 6A).** WT PC-3 cells-derived tumors weighed significantly more than tumors developed from Cat$^{+/-}$ tumors (**Fig. 6B)**. Also, WT PC3 cells-derived tumors had significantly larger volume than those developed from Cat$^{+/-}$ cells (**Fig. 6C).**

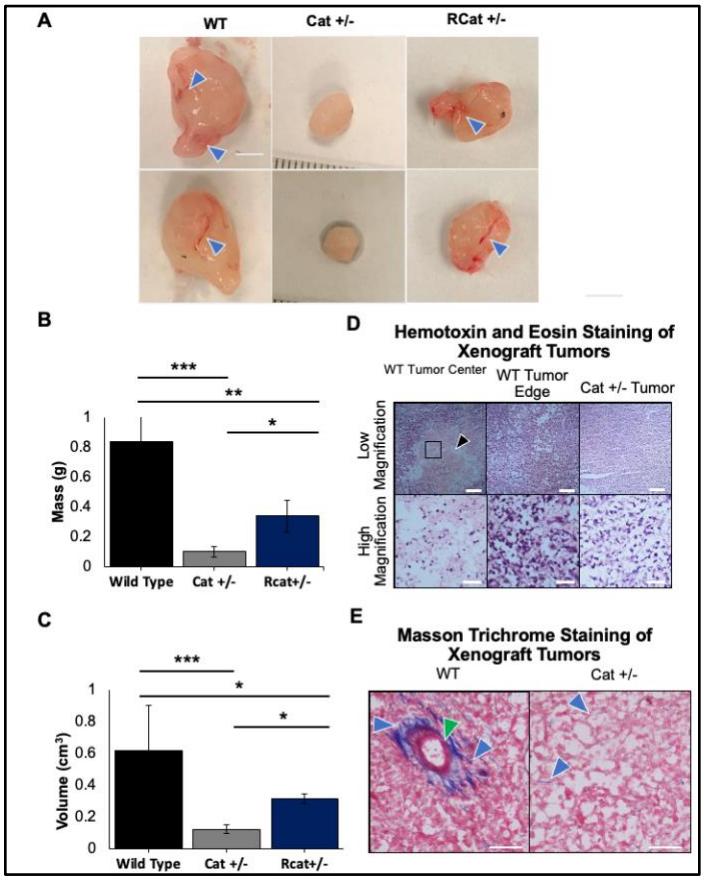

Figure 6. Catalase knockdown cells form smaller xenograft tumors and produce less collagen. When Cat$^{+/-}$ cells were Xenografted into immunodeficient SCID mice, they formed significantly smaller tumors. (**A**) Representative images of tumors. (**B**) Quantification of tumor mass. (**C**) Quantification of tumor volume. All data are Mean ± Standard Deviation *= p< 0.05, **=p<.01, **=p<.001. Tumors, Cat+/- n = 5, WT n = 4 RCat+/- n=5. Scale bar is 0.5 cm. Histological stains of xenograft tumors. Arrowheads show necrotic tumor center. Representative section of xenografted tumors are stained with Hematoxylin and Eosin (**D**) showing that the Cat$^{+/-}$ tumors are solid whereas the WT tumors show signs of cell death indicated by the black arrowhead. (**C**) Masson Trichrome staining of tumor sections show that Cat$^{+/-}$ xenografts contains less collagen indicated by blue arrowheads and fail to establish blood vessels as is the case with the WT tumors (green arrowhead). Scale bars are 100 and 50µm for lower and higher magnification, respectively.

To determine whether the decrease in tumor size generated by the $Cat^{+/-}$ cells was due to catalase loss, we performed xenografts experiments using the $RCat^{+/-}$ cell line and observed that the $Rcat^{+/-}$ -derived tumors grew to a size between that of the tumors caused by the WT cells and the $Cat^{+/-}$ cells (**Fig 6A).** We also observed that the presence of blood vessel formation was similar to that of the WT xenografts. Xenografted tumors were also sectioned and stained with Hematoxylin and Eosin. $Cat^{+/-}$ tumors were more solid, whereas WT tumor showed evidence of necrosis in the center of the tumor (**Fig. 6D**, arrowhead). In addition staining with Masson trichrome blue, showed that the $Cat^{+/-}$ tumors have very little collagen staining comparing with WT PC3 tumors (**Fig. 6E**, arrowheads).

DISCUSSION

When castration-resistant prostate cancer becomes metastatic there is a poor prognosis and therefore there is currently a need for novel therapeutic targets. As of right now, there is no reliable treatment for mCRPC. Drugs currently used at this stage of disease (such as enzalutamide and cabazitaxel, often called “third line treatments") have severe side effects that can lead to discontinuation of use as well as a generally poor impact on serum PSA and overall survival [27,28]. This points to the need for thinking outside of the typical Androgen-Receptor pathway for new drugs. In the work presented here, we identify the antioxidant enzyme, catalase, as a promising target for prostate

cancer management. We used a cell line that is not only hormone-refractory but is also metastatic (derived from a metastasis in the human bone) [https://www.atcc.org/products/crl-1435] and an *in vivo* xenograft model to investigate the role of catalase in prostate cancer development. The sensitivity of tumor cell lines to ROS has been well characterized [29]. Prostate cancer cells specifically have been shown to be more sensitive to H_2O_2 mediated apoptosis [16,17] leading us to consider catalase, an H_2O_2 scavenger, as a potential target for anti-cancer treatment.

In order to characterize prostate cancer progression with lower catalase activity, we established a CRISPR-Nickase (NIC)-mediated catalase knockdown in the mCRPC cell line (PC3). After confirmation of a four base-pair DNA deletion at the CRISPR gRNA target site in the catalase gene and of a decrease in catalase activity, we determined that this catalase knockdown cell line ($Cat^{+/-}$) was a good model to study tumorigenicity of mCRPC when catalase is suppressed. Consistently, we found that lower catalase expression resulted in reduction of processes critical for prostate cancer progression. We show that the mCRPC cell line deficient in catalase ($Cat^{+/-}$) in *in vitro* studies has: (i) 50% less catalase activity; (ii) reduced cell proliferation and migration; (iii) decreased cell adhesion to collagen; (iv) increased cell adhesion to matrigel, a basement membrane-like matrix; and (v) has much reduced growth *in vivo* during tumor development resulting in highly significant tumor volume, tumor weight and lack of blood vessels.

Increased cellular proliferation is a major hallmark of cancer [4]. We found that the $Cat^{+/-}$ cells proliferate slower than the wild-type cells. The rescue experiment confirmed that this decrease in proliferation is due to catalase loss. We also confirmed that the $Cat^{+/-}$ cells are half as sensitive to H_2O_2 as WT PC-3 cells**.** Given that WT PC-3 cells grow nearly as slowly as $Cat^{+/-}$ cells in the presence of 25μM of H_2O_2, it is likely the $Cat^{+/-}$ cell line has lower proliferation in response to H_2O_2 accumulation. Consistent with what has previously been shown in other cancers, this is likely because cancer cells are already more sensitive to H_2O_2 [15-17]. Decreasing their ability to detoxify the microenvironment, will lead to an increase in cell death. Given that cells produce their own H_2O_2 and that cancer cells are known to produce more H_2O_2 than their tissue healthy counterparts [30], lowering catalase activity can lead to tumor destruction.

In addition to proliferation being an indicator for cancer progression, the ability to metastasize is an important hallmark of cancer [14]. One of the major steps in metastasis is the ability of the cancer cells to migrate through collagen-rich connective tissue towards blood vessels, adhere to the basement membrane, and enter blood vessels to travel to distant sites where the process is reversed. Our finding that $Cat^{+/-}$ do not adhere well and migrate much slower than the WT PC3 cells indicates that loss of catalase interferes with the expression of adhesion molecules, which are important in cell and molecular mechanisms of metastases. Because we were able to rescue the adhesion by re-

expressing catalase, we know that it is catalase loss that is contributing to this phenotype.

Clinically, prostate cancer tumors exist in a three-dimensional environment therefore, we tested the ability of the $Cat^{+/-}$ cells to form solid tumors in a xenograft model. We found that $Cat^{+/-}$ cell tumors were significantly smaller in weight and volume compared to WT tumors, indicating that catalase loss leads to significant inhibition of tumor growth *in vivo*. Not only did catalase suppression lead to lower proliferation of the cancer cells in culture, but it also slowed the growth of a solid tumor. Furthermore, the $Cat^{+/-}$ tumors also had fewer blood vessels, decreasing their ability to obtain O_2 and nutrients needed to progress aggressively or metastasize. Angiogenesis is a major hallmark of cancer [4,31]. Although the tumors-derived form our rescue $Cat^{+/-}$ cells ($Rcat^{+/-}$) did not reach the same size as WT, they did have the increase in blood vessels we see in WT tumors. Decreased angiogenesis is typically associated with less aggressive cancer because tumor cells are unable to maintain the nutrients needed for sustained growth and proliferation, and have fewer microvessels available to metastasize [4,31]. Upon further investigation, we found that our rescue plasmid does not overexpress catalase in the tumor xenograft model to the same extent it does in cell culture conditions (data not shown).

Staining of tumor sections showed that the $Cat^{+/-}$ cells-derived tumors have much less collagen than the WT PC3 cells-derived tumors. High collagen density in tumors is generally associated with poor prognosis [32,33] therefore a

lower density of collagen is a characteristic of a less aggressive tumor. High amounts of collagen also can contribute to hypoxic conditions in tumors, signaling increases in aggression and angiogenesis [32,33]. Recent studies have shown that higher levels of collagen in tumors result in lower T-cell infiltration in breast cancer, so reduction in collagen may be even more important in cancer management than previously thought [32,33].

In conclusion, we show here that catalase is a potential target for Metastatic Castration-Resistant Prostate Cancer. Stable knockdown of catalase results in slower-growing cancer cells that are more susceptible to H_2O_2 mediated cell death. Catalase deficient tumor cells show several phenotypes of a cancer that will migrate and metastasize very poorly. We are currently investigating the impact that catalase loss has on adhesion to different cell culture substrates. Overall, catalase reduction leads to suppression of progression of Prostate Cancer in both *in vitro* and *in vivo*.

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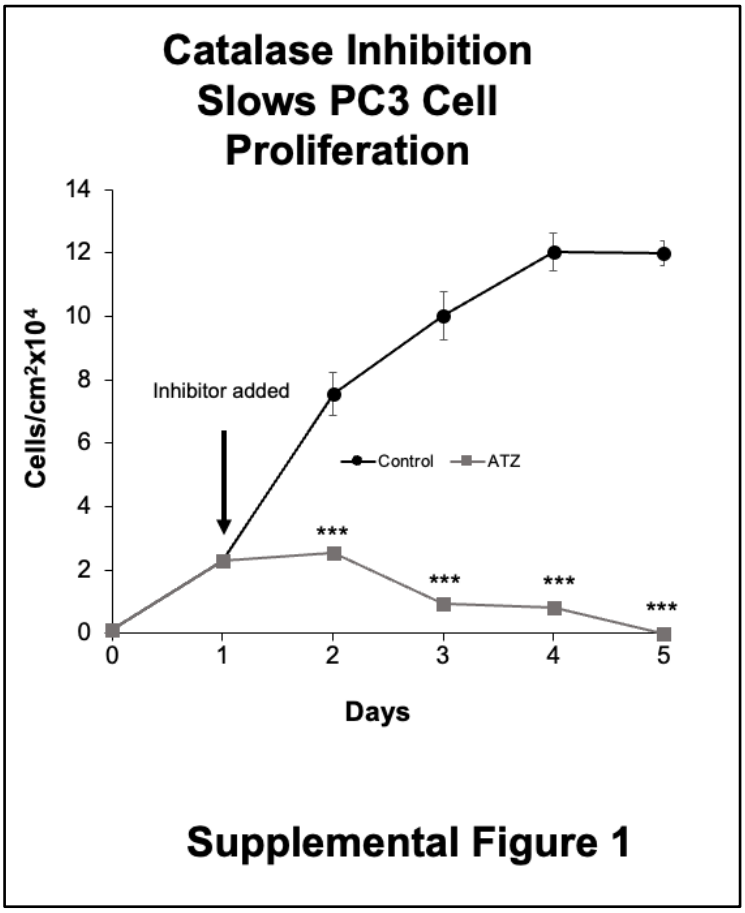
SUPPLIMENTAL FIGURES



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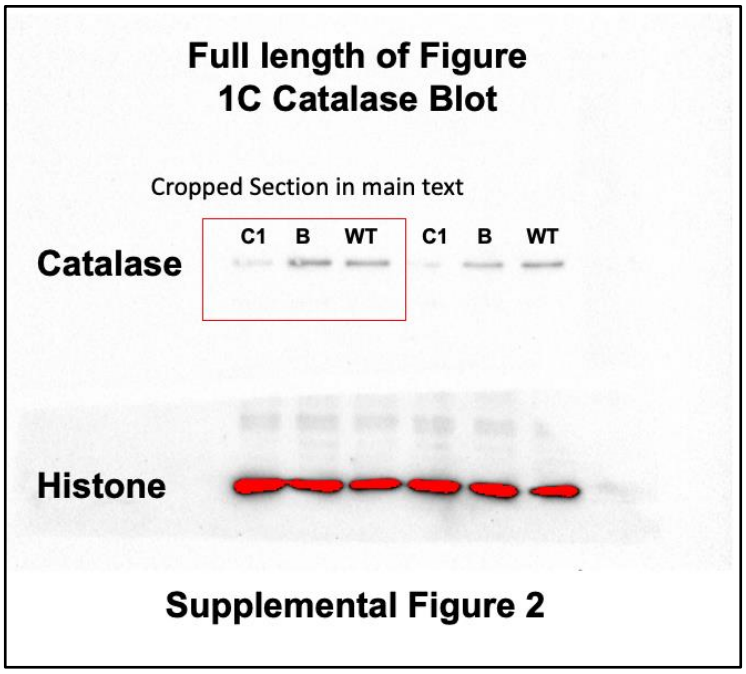
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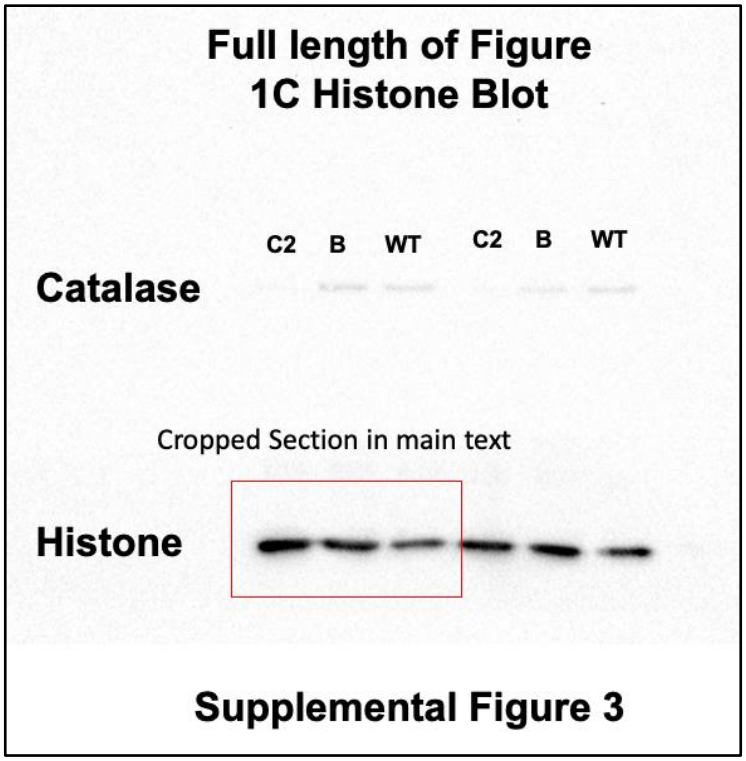
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Chapter 3: Third Hand Smoke Causes Epigenetic Alterations to Mitochondrial DNA

Abstract

Although cigarette use has declined in the United States in recent years[1], potentially reducing human exposure to First Hand Smoke (FHS) and Second Hand Smoke (SHS), the dangers of Third Hand Smoke (THS) still remains underappreciated to the general public. Third hand smoke has previously been shown to cause non-smoking persons to test positive for a smoking metabolite when subjects are exposed to it. Controlled mouse studies have shown that metabolism in energy demanding tissues like the brain, muscle, and liver is severely altered. Fatty oxidation is impaired in the liver leading to an increase in fat deposits ATP production is decreased in many tissues when a mouse is exposed to THS. All this evidence points to THS impacting mitochondrial health. Mitochondrial DNA (mtDNA) encodes some of the proteins that are used in the electron transport chain (ETC) and methylations in mtDNA have been implicated in causing metabolic dysfunction. In this study, we exposed C57/bl6 mice to THS using a Teague smoking machine. After 4 months of exposure, the mtDNA in their liver was isolated and analyzed for methylations and mutations compared to another mouse with no THS exposure. We found that there is an increase in relative mtDNA methylation, indicating that THS impacts mitochondrial epigenetics. The evidence shown in this study supports the hypothesis that THS causes mitochondrial dysfunction in part via epigenetic alterations to mtDNA.

Introduction

Despite organized efforts to curb the impacts of cigarette smoke, tobacco continues to cause health problems worldwide. Approximately 20% of cancer deaths worldwide is due in some part to tobacco exposure[2]. Smoking also costs time and money. In 2018 the CDC estimated that $300 Billion is spent on smoking related illness. It was also reported that $5.6 billion was lost productivity due to secondhand smoke exposure[3]. The dangers of FHS and SHS are widely recognized and publicly accepted. When scientific data showing SHS was found to be even more toxic than FHS[4], it gave rise to smoking bans in public spaces across the world. More recently, data is emerging that there is a third danger that comes from tobacco smoke. We now know that SHS can settle in and contaminate various surfaces in the home. Once exposed to light and oxygen, the toxins from the SHS can react and become even more toxic[5]. This type of tobacco-derived pollutant is called Third hand smoke (THS).

Until recently, THS was an underestimated toxin, which was quietly impacting human health[6]. THS can be defined as the toxins that accumulate on surfaces that are exposed to secondhand tobacco smoke (SHS). A small handful of the chemicals that have been detected in THS include: hydrogen cyanide (used in chemical weapons), butane (used in lighter fluid), toluene (found in paint thinners), arsenic, lead, carbon monoxide and polonium-210, a highly radioactive carcinogen[2]. A particularly insidious aspect of THS is that many of the people who are exposed to THS are unaware of it. THS can be a contamination of rental

properties (especially apartments that lack ventilation, rental cars, used cars, and older homes. In homes with known SHS exposure, a study was conducted to see how much THS toxins were deposited in the house. Nicotine in dust averaged 64.0 μg/m^2 in living rooms and 15.8 μg/m^2 in infants' bedrooms[7]. Infants and toddlers are expected to be of increased risk when exposed to THS because of their smaller weights and higher likelihood of ingesting the dust when crawling or learning to walk. It was even found that THS can even remain in the environment two months after smokers leave the residence. In this study, homes were vacant for two months, some even cleaned and repainted, yet still have THS present when the new tenants moved in[8].

To combat the health impacts of THS, we must collect the evidence of how THS impacts health, what groups THS impacts the most, and use this data to advocate for policy changes that prevent homes from being contaminated. THS is a public health risk, but it is difficult to separate out the impacts of THS, FHS, and SHS. For example, the biomarker cotinine shows up in the serum of those exposed to THS, but it also is in the serum of those exposed to FHS and SHS. This creates the need for two major tools in studying THS: 1) An experimental setup that can model THS without the risk of FHS or SHS contamination 2) find biomarkers that occur in response to THS but not SHS. As described in Jacob et al[9], there is one class of chemicals that is prevalent in THS in higher concentrations than SHS and FHS, tobacco specific nitrosamine (TSNA). This could lead to the formation of specific DNA adducts of TSNA that

can be used as biomarkers.

It has been shown that exposure to THS leads to various metabolic issues, including increased risk of fat accumulation in liver[6] as well as diminished ATP production[6,10]. THS exposure also impacts wound healing[6]. Despite many of the impacts of THS being observed, the mechanism of which these impacts occur is still mostly unknown. Interestingly, in mice, many of the negative impacts of THS will dissipate after living in a clean environment for 3 to 6 months. ATP production along with other markers of mitochondrial health, however, do not return to normal. Therefore, the mechanism at which ATP production is slowed is both unknown and permanent.

Since THS has been shown to induce biomarkers of general DNA damage[10] we hypothesized that some of this DNA damage is occurring in the mitochondrial DNA (mtDNA) which would lead to perturbed ATP production via damage to the DNA encoding the various complexes of the electron transport chain. We also hypothesize that epigenetics of mtDNA is impacted. It is known that FHS increases the frequency of DNA methylation in the genome as a whole[11] and that hypermethylated mtDNA is generally associated with poor health outcomes[12,13].

In our studies, we tested 5mC levels in mtDNA to determine if THS was causing metabolic problems via increased mtDNA methylation. In summary, demonstrate genetic and epigenetic impacts of THS on mtDNA and further our understanding of THS impacts. This allows us to provide evidence for legislative

changes that will reduce the amount of people exposed to THS.

Materials and Methods

***Isolation of mitochondria*:** Briefly, 50mg of **liver** tissue will be ground in liquid nitrogen using a mortar and pestle to a fine powder. This will be subject to bead homogenization (5 Min speed 6) in 500µl of TEK Buffer (0.05 M Tris, 0.01 M EDTA, 1.5% KCl (w/v), pH 7.5). TEK was added to bring lysates up to 2ml. Lysate was centrifuged for 10 minutes at 800 x g to pellet the nuclei. Mitochondria-containing supernatant will be centrifuged at 4ºC for 30 minutes at 15,000 x g. Then, the mitochondrial pellet was resuspended in TEK buffer and centrifugation was repeated to increase purity. Crude Mitochondrial pellets can be frozen in liquid N_2 and stored at -80ºC or processed further for purification of contents.

Isolation of mtDNA: Mitochondrial pellet was lysed in 400µl of lysis buffer with 20µl of proteinase K and left to incubate at 55ºC for **at least** one hour. When the solution became clear (1-3 hours) it was centrifuged for 5 minutes at 15,000 x g to pellet any remaining cell debris. 100µl of Potassium acetate to precipitate our SDS from lysis solution. 500µl of 24:1 Chloroform: Isoamyl alcohol was added, and tubes were shaken for 20s. Mixture was then centrifuged for 10 min at 15,000 x g. The aqueous half was removed and transferred to the new tube. DNA was precipitated by adding 2x the volume of Ethanol to the lysate and incubated overnight at -20ºC. DNA was pelleted by centrifugation at 15,000 x g

for 10 minutes, allowed pellet to air dry for 5 min and resuspended in distilled water.

5mC/5hmC Specific ELISA. We will use an antibody-based method to quantify percent methylation. MethylFlash Global DNA Methylation (5-mC) ELISA Easy Kit (P-1030-48) is an ELISA-based assay. 100ng of mtDNA is bound to a treated 96-well plate along with a positive control hyper methylated DNA and negative control unmethylated DNA. An anti-5-mC antibody is added to the plate, incubated at room temperature for 50 minutes, washed and treated with a color developer that can be read spectrophotometrically at 450 nm.

Statistics. Differences between groups were assessed using a student's t-test in Graphpad Instat Software (Graphpad, La Jolla, CA, USA). Data is represented as mean +/- Standard Deviation represented as error bars.

Results

Isolation and Verification of mtDNA Isolation

To do mtDNA mutation analysis, its necessary to first verify that the extracted mtDNA has high purity. mtDNA sequencing and methylation detection can be influenced greatly if there is nuclear DNA (nDNA) contamination.

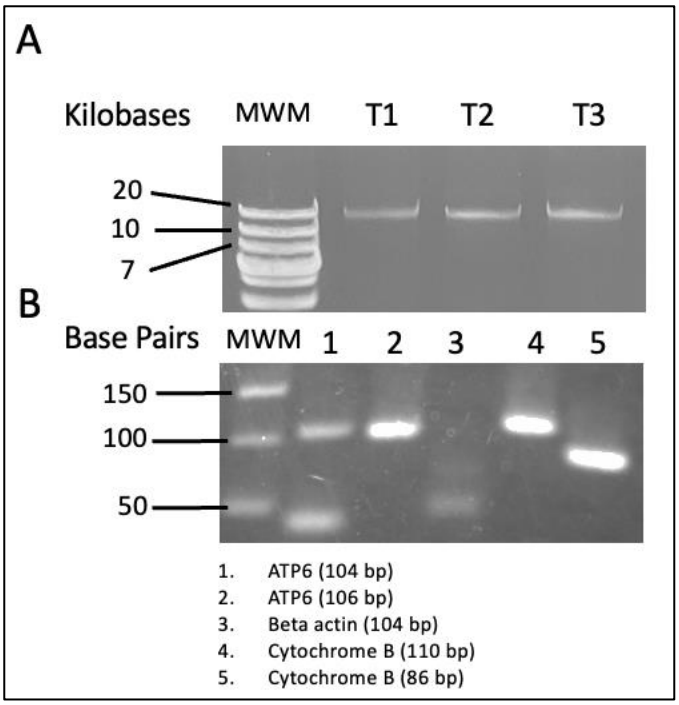

Figure 1. Verification of mtDNA purity. (**A**) The mtDNA samples run on a 1% agarose gel with a molecular weight marker (MWM) **(B**) PCR Products with mtDNA used as a template demonstrating there is not enough nDNA

We evaluated the mtDNA by both size and PCR. mtDNA that was extracted was first confirmed by gel electrophoresis (Fig.1A). We found that because mtDNA can supercoil, it forms bands at different molecular weights like plasmid preps. The mtDNA was also tested via PCR amplification of two regions of the mitochondrially encoded ATP6 and Cytochrome B genes (Fig. 1B). All four regions of mtDNA that we amplified had a band of the correct size and the amplification of beta actin was unsuccessful. From this we were able to verify that we have pure mtDNA with little nDNA contamination.

THS Exposed Mice have an Increase in 5-Methyl Cytosine (5-mC)

In general, a higher state of oxidative stress is associated with higher levels of DNA methylation. DNA methylation can have a repressive effect of genes like mutations. To begin to assess the mitoepigenome, we used an antibody-based approach to quantify the relative methylation in mtDNA from THS-exposed mice. We found that in both females (Fig. 2A) and males (Fig. 2B), relative mtDNA methylation was increased when exposed to THS. We found that in males however, the difference in methylation between THS and control are larger.

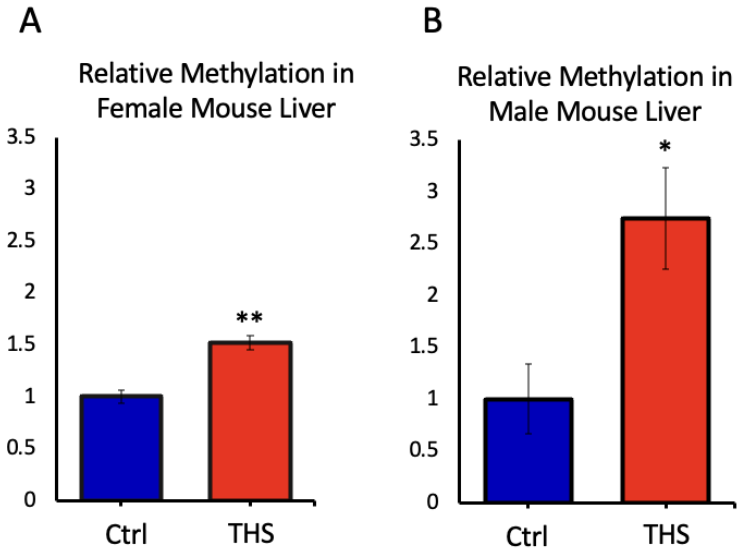

Figure 2. Methyl-ELISA Shows THS causes an increase in 5mC across sex A) ELISA Results demonstrating that male mice exposed to THS have more relative 5mC **B)** ELISA Results demonstrating that female mice exposed to THS have more relative 5mC

Discussion

Despite the impacts of THS being historically underrepresented, it has demonstrated the potential to cause long term damage to human health. In humans, many people who are exposed to THS are exposed to SHS, so it would be extremely difficult to disentangle the effects of the two without a controlled environment. By studying THS on a mouse model in a controlled environment, we were able to separate the effects of THS exposure from the effects of SHS and study the effects of THS specific toxins.

Past literature showed **what** biological effects THS had, where this paper sought to determine the **how** behind those effects. THS has been shown to decrease ATP production in the liver and the brain[10] as well as increase lipid accumulation in the liver[6,10]. Because many of the impacts of THS are associated with metabolism, it was likely that THS was impacting the mitochondria, much like FHS, SHS, and air pollution have been known to do. Both FHS[14] and air pollution[13,15] have been known to cause similar metabolic symptoms that coincide with an increase of mtDNA methylation. Because of the many similarities in the compounds found in SHS, pollution and THS, we hypothesized that mtDNA would also exhibit an increase in mtDNA methylation and mtDNA mutations.

A Teague smoking machine was used to expose household fabrics to SHS. The fabric was then removed from the smoke machine and placed in the mouse's cage. The mice were exposed to THS for 4 months, sacrificed, and

livers were collected. Because DNA methylation is known to increase with age, it was important that the clean air controls were the same age as the mice exposed to THS. Control mice were sacrificed at the same ages as the THS exposed mice. The mtDNA from the liver was isolated from both groups to assess for mutations and methylations.

It is essential to isolate mtDNA before sequencing, rather than relying on bioinformatics to match specific mutations or methylations back to mtDNA. In many organisms, there is a transfer of a small amount of mtDNA to the nuclear genome, which can be incorporated. These regions of mtDNA sequences in the nuclear genome are called nuclear copies of mitochondrial DNA (NUMTs)[16]. According to a study that used BLAST to search for NUMTs within different organisms, mice have been known to have about 37.67 kB of NUMT regions in their genome, whereas the human genome has about 263.47 kB of NUMTs content[17]. Although the number of NUMTs in the genome is small compared to the total size of the genome, mtDNA abundance is also small compared to the total size of the genome. nDNA contamination containing NUMTs have the potential to bias sequencing results. For example, mtDNA lacks histones and is exposed to more endogenous oxidative stress generated by the ETC. These two circumstances lead to mtDNA being more prone to mutations than its NUMT counterparts might be, making NUMT contamination distort the results.

nDNA contamination in an mtDNA prep is particularly problematic during assays that detect methylation. The nuclear genome has a much higher rate of

methylation than the mitochondrial genome, so nDNA contamination can lead to a methyl-ELISA assay to overestimate the amount of 5mC in the mtDNA sample.

To combat the possibility of nDNA contamination in the mtDNA preparation, mtDNA was isolated from nDNA in two ways. First, the entire mitochondria is separated from the entire nuclei via centrifugation. Mammalian nuclei are large compared to mitochondria and can be pelleted by centrifuging at a very low speed. Mitochondria, however, need to be centrifuged at high speeds for long periods to pellet out of solution. This difference in mass allows for the mitochondria to be isolated before the DNA extraction. The only potential nDNA contamination that can remain in sample are from nuclei that prematurely lysed during centrifugation. Next, to remove any nDNA carryover, the mtDNA was run on an agarose gel, and the 16kb band was cut out before verification was performed. A PCR was done to verify that the DNA collected was mostly mtDNA. Two genes in the mitochondria were targeted for amplification with two different primers. The result showed that the DNA we isolated was mtDNA. A PCR of a nuclear gene was performed to check for contamination, and nothing was able to be amplified, which indicates that the mtDNA was pure enough to continue sequencing and analysis.

Mitochondrial DNA methylation has been a new area of interest amongst scientists. Until recently, the existence, impact, and physiological relevance of 5mC in mtDNA was hotly debated[25]. However, advancements in sequencing technology have allowed scientists to demonstrate that mitochondrial methylation

does indeed occur in mammals[26], and some studies have even started to link the mtDNA methylations to changes in gene expression[27]. It is possible that the authors on the earlier paper believed that mitochondrial DNA methylations had little impact on gene function, because in the nuclear genome, CpG methylations are known to be repressive, but in mtDNA, there is evidence suggesting that GpC methylation is what can influence gene expression[27]. Aberrant mtDNA methylation has since been associated with several diseases including: Cardiovascular disease[12], Parkinson's disease[28], body composition [29], ALS[30], FHS exposure[19], air pollution exposure[18] and breast cancer[31]. From our findings, THS can be added to this list as well.

The mtDNA of mice that were exposed to THS did have an overall increase in mtDNA methylation, as predicted. Oxidative stress is associated with an increase in DNA methylation[32]. This evidence proves that THS leads to genetic and epigenetic changes in mtDNA, but our evidence does not prove with certainty that these epigenetic alterations are causing the effects we see (decreased ATP production, increased fatty tissue). It is possible that ROS in THS toxins are impacting function of metabolically related proteins post-translationally and causing an increase in mtDNA methylations. Cell culture studies have pointed to a mechanistic connection between mtDNA methylation and gene expression, but this has yet to be repeated in animals[27]. It is still possible that these DNA methylations are causing the phenotype we see, but it is difficult to provide a causal link because tools that allow us to precisely control

methylations are not as advanced as the tools that allow us to study mutations.

For example, if a gene is thought to be involved in a specific disease process, that gene can be mutated or knocked out, a phenotype can be observed. A rescue can be performed to confirm that the phenotype observed is due to the mutation rather than an off-target effect of the tool used to mutate or edit the DNA. The molecular tools that are being developed to add methylations to DNA have not become this precise, yet. One lab is very close to developing a tool that could revolutionize epigenetics. They used a catalytically dead CRISPR-cas9 protein that was attached to a catalytically active methyltransferase enzyme to add methylations to a section of DNA. This does not allow one to pick specific cytosine bases to methylate, but it will generally increase the number of methylations in a 35 base pair wide section[33]. This tool may be useful for testing the effect of increased methylations in promoter regions, or even the mitochondrial D-loop, but more precise tools are needed to study the effects of individual methylations. As more precise tools for studying epigenetics become available, the effect that THS has on the mitochondrial genome methylation should be reproduced without the exposure, so we can observe what specific impacts the methylations by themselves were responsible for. The development of more precise molecular tools will likely lead to us uncovering more effects that THS has on the mitochondrial genome and epigenome.

In conclusion, it seems that THS causes even more problems than what has been previously thought. It is important to continue to monitor how THS

could potentially impact human health because it allows us to better inform both the public, and legislators of the dangers so public health measures can be taken to prevent more THS exposure. If THS continues to accumulate as a contaminant in the environment, it has the potential to cause metabolic harm to the members of the community it contaminates.

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Chapter 4: Closing Remarks

PCa is a serious health threat in the United States with 34,700 PCa related deaths predicted to occur in 2023. Although the number of men diagnosed with PCa *seemingly* decreased between 2007 and 2014, this has since been attributed to changing in screening protocols which lead to underdiagnosis, nationally. Since 2014, PCa incidence has continued to steadily increase [1]. There are different presentations of PCa that impact treatment. ADPC typically responds well to treatments that lower the amount of circulating androgens in the body. A persistent side effect of the drugs that achieve this, is increased oxidative stress[2]. Because oxidative stress is known to be essential for the progression of CRPC, in many patients, the PCa will inevitably progress to the CRPC phenotype. Once PCa is castration resistant, first and/or second-generation anti-androgen drugs can be used but each one has associated drug resistance[3]. There is a prostate cancer vaccine called sipuleucel-T (provenge) but it only increases survival for an average of about 4 months[4]. Once second-generation anti-androgen drugs fail, there is not much that can be done for patients. Therefore, more research is needed for novel drug targets to treat ADPC.

Catalase suppression has already been shown to slow proliferation of another hormone-driven cancer, breast cancer[5]. Most of these studies, however, were done using cell culture. Although it provides compelling support, treating cancer cells that are adhered to a flat tissue culture dish does not fully model

how cancer cells behave when making up three-dimensional tumor tissue. A CRISPR-based molecular tool was used to generate a catalase knockdown in a human tumor cell line derived from a patient with ADPC. The benefits of making a knockdown cell line were that it could also be used to create a tumor xenograft. The knockdown was confirmed to be stable via several molecular detection methods including qPCR and western blot. Next, the candidate cell lines were tested for catalase activity. A cell line that exhibited 50% transcription lower and 50% lower activity compared to wildtype was selected for testing. A PCR of the region around the CRISPR cut site confirmed disruption of the gene. The lower catalase activity in the cell line confirmed that the disruption in the gene sequence led to the disruption in the function of the protein.

It was found that the tumor line that expressed 50% less catalase activity (Cat+/-) also exhibited twice the sensitivity to hydrogen peroxide treatment compared to the parent cell line (WT). The Cat+/- cell line also proliferated about twice as slow in vitro. Slower proliferation in vitro can indicate that the cell line would also grow slower in tumor form. They also exhibit lower adhesion capabilities. Adhesion is very important for cancer cell migration, and alterations in adhesion can hinder a cell's ability to migrate and metastasize. To look at migration more closely, we used an in vitro assay called a scratch assay which looks at the cells migration over time to close a “scratch” on the tissue culture plate. Cat+/- cells also migrated and closed the gap much slower than WT cells.

This suggests that they likely would not metastasis as effectively in vitro compared to WT.

Although some in vitro data supporting catalase's capability to be a cancer target exists, very few tests have been done in the three-dimensional tumor environment. Xenografting human cells into an immunodeficient mouse is an appropriate model for this. Using human cells is beneficial because the goal of these studies is to find a relevant target for the development of therapies to treat human cancer. Using mice allows us to see if targeting catalase holds up to targeting a whole tumor, rather than cells on a plate. Incredibly, the Cat+/- cells formed tumors that were on average much smaller than the tumors formed by WT cells. This indicates that catalase could be a potential drug target in combating prostate cancer.

Catalase is an important enzyme in controlling oxidative stress, so targeting it with a drug for potential therapies will not be simple. Many existing inhibitors of catalase like hydrogen sulfide, sodium azide, hydroxylamine, and potassium cyanide are toxic to humans at very low levels[6]. There is one catalase inhibitor that is relatively less toxic, 3-amino-1,2,4-triazole (ATZ), which was shown to slow the growth of PCa cells in chapter 2. New ATZ derivative compounds have recently been synthesized and screened on various cancer cell lines that were then tested for viability[7]. Although prostate cancer was not included in the screening, based on our CRISPR results, these suppressing compounds would have likely been successful if tested on CRPC cells, and

future studies should include screening these compounds for their capability to selectively kill prostate cancer cells. Overall, with the evidence demonstrated here and by the literature, catalase stands as a potential candidate to be targeted for by a new prostate cancer drug.

It has been described how increasing intracellular ROS via catalase suppression can be used to target prostate cancer. On the flip side, too much ROS can also lead to health problems. Environmental sources of ROS like pollution or tobacco exposure can cause DNA damage. Although the link between poor health outcomes and exposure to: pollution[8–10], first hand tobacco smoke[11,12], and second hand tobacco smoke[13,14] have all been well documented, the link between third hand smoke exposure and poor health outcomes has only been appreciated more recently. Although some of the impacts of THS have been documented, the entire mechanism at which these impacts occur has yet to be identified.

Third hand smoke is finally gaining some public attention thanks to the research being done on the extent of its health effects. THS has been shown in the literature to cause alterations in the processes for blood clotting[15], wound and healing[16]. More recently, THS has been shown to alter metabolic processes in mice. For example, one study found that THS exposure leads to an increase in lipid accumulation in the liver, higher fasting serum insulin and fasting glucose levels[17]. THS exposure has also been shown to lead to lower ATP production in the liver, brain, and muscle tissue of exposed mice[18].

ATP reduction and an increase in fat deposits in the liver point to a problem in the mitochondria. The connection between metabolic problems and tobacco use are well established[19]. SHS exposure has been shown to alter liver metabolism, leading to steatosis[20]. Smoking mothers have been shown to have an increase in mtDNA methylation in the placental tissue[21]. Ambient air pollution, which has similar compounds to THS, has also been shown to increase mtDNA methylation[22–24].

Mitoepigenetics, is a new term for a quickly growing topic in epigenetics. Due to a bacterial ancestor, our mitochondria have their own DNA. Some of the components for the electron transport chain are encoded on mtDNA. mtDNA does not have a histone to epigenetically regulate it. Although mtDNA can be methylated, past, accurate methyl-sequencing methods often led to mischaracterization of the mitochondrial epigenome. Because of this, mitochondrial epigenetics has been historically underappreciated. Even as recently as 2017 a paper was published suggesting that they have evidence of the absence of mtDNA methylations[25]. Despite the controversy, the increased accuracy of bisulfite sequencing allows researchers to gain a clearer understanding of how methylation of mtDNA can impact different tissues metabolically.

Despite the initial controversy, we now know that mitochondrial epigenetics has a curious role in human health and Mitoepigenetics has become a rapidly developing field. Innovations in bisulfite sequencing technology has

allowed us to study the effect that methylation of mtDNA can have in disease. For example, a recent study found that with mtDNA methylation studies having implications in amyotrophic lateral sclerosis (ALS)[26], cardiovascular disease[27], cancer[28], and response to environmental toxins[22–2421].

We found that in the liver tissue of THS exposed, mtDNA had a higher proportion of methylated cytosines, consistent with our hypothesis. Future studies could seek to locate the exact locations of the methylations using bisulfite sequencing. Bisulfite sequencing is a technique that chemically or enzymatically converts Cytosine in a DNA sample to Uracil. If a Cytosine is methylated however, it remains intact. At the end of the bisulfite conversion process, the DNA can then be sequenced, and the locations of 5mC can be determined by comparing the sequence to an untreated sample of the same DNA.

In conclusion, we are only beginning to understand mitochondrial DNA methylation and therefore only beginning to understand one of the known effects of THS. As tools to study DNA methylation are continuing to become more precise, we will be able to gather even more information about if and how the increased methylation and mutations we see in THS exposed mice contribute to the phenotype we see.

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