August 2015

Antioxidant Function of Pigment Epithelium Derived Factor in Adipose Tissue, the Prostate, and Prostate Cancer

Lyndsey Sandra Crump
University of Wisconsin-Milwaukee

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ANTIOXIDANT FUNCTION OF PIGMENT EPITHELIELUM DERIVED FACTOR IN ADIPOSE TISSUE, THE PROSTATE, AND PROSTATE CANCER

by

Lyndsey Crump

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biomedical Sciences at The University of Wisconsin- Milwaukee

August 2015
ABSTRACT

ANTIOXIDANT FUNCTION OF PIGMENT EPITHELIUM DERIVED FACTOR IN ADIPOSE TISSUE, THE PROSTATE, AND PROSTATE CANCER

by

Lyndsey Crump

The University of Wisconsin- Milwaukee, 2015
Under the Supervision of Jennifer A. Doll, PhD

Aggressive prostate cancer (PCa) is positively correlated with obesity and a high fat diet, suggesting that dysregulated lipid metabolism promotes PCa progression. Pigment epithelium-derived factor (PEDF) regulates angiogenesis, lipid metabolism, and has antioxidant function in other cell types. In the prostate, PEDF inhibits angiogenesis, and its expression is decreased in PCa. However, PEDF’s role in regulating lipid metabolism and oxidative stress levels has not been investigated, and, as such, was the goal of the present study. Oxidative stress levels were evaluated in vivo and ex vivo in prostate and adipose tissues from wildtype (C56Bl/6J) and PEDF knockout (KO) mice. In vitro, human normal prostate or PCa cell lines were treated with PEDF with or without oleic acid (lipid overload model). Reactive oxygen and reactive nitrogen species (ROS/RNS) and antioxidant enzyme levels (GPx-3 and mSOD) were quantified using an ROS/RNS detection assay and Western blot, respectively. Loss of PEDF in adipose tissue increased ROS/RNS, indicating elevated oxidative stress. PEDF loss also decreased GPx-3 and mSOD levels in the adipose tissue. However, these results were not seen in the prostate or in PCa cells. While these data do support the hypothesis that PEDF has some
antioxidant function in adipose tissue, further studies are needed to elucidate this role.
# TABLE OF CONTENTS

List of Figures...........................................................................................................v
List of Tables.............................................................................................................vi
List of Abbreviations..............................................................................................vii
Acknowledgments ...................................................................................................ix
INTRODUCTION.........................................................................................................1
  Prostate Cancer.....................................................................................................1
  PCa Diagnosis and Treatment..............................................................................2
  PCa Risk Factors.................................................................................................5
  Obesity and PCa.................................................................................................7
  Pigment Epithelium Derived Factor.................................................................11
  PEDF’s Role in Tumor Progression....................................................................14
  PEDF and Metabolism.......................................................................................16
  PEDF and Oxidative Stress...............................................................................18
  Summary and Gap in Knowledge.....................................................................20
  Hypothesis and Specific Aims..........................................................................21
MATERIALS AND METHODS ..............................................................................22
RESULTS ..................................................................................................................30
DISCUSSION ............................................................................................................46
CONCLUSIONS AND FUTURE DIRECTIONS....................................................61
REFERENCES ..........................................................................................................63
LIST OF FIGURES

Figure 1. Loss of PEDF increases oxidative stress in adipose but not prostate tissue.................................................................31
Figure 2. PEDF loss exhibits no significant change on GPX-3 and mSOD levels in adipose tissue.........................................................32
Figure 3. PEDF loss exhibits no significant change on GPX-3 and mSOD levels in prostate tissue..........................................................32
Figure 4. Loss of PEDF exhibits no significant change on oxidative stress in response to H$_2$O$_2$ stimulus in adipose tissue......................33
Figure 5. Loss of PEDF exhibits no significant change on oxidative stress in response to H$_2$O$_2$ stimulus in prostate tissue......................34
Figure 6. PEDF treatment has no effect RWPE-1 cell viability or ROS/RNS.............................................................................36
Figure 7. PEDF treatment does not significantly affect mSOD or GPX-3 expression in RWPE-1 cells in an in vitro obesity setting..............37
Figure 8. PEDF treatment has no effect LNCaP cell viability or ROS/RNS.............................................................................39
Figure 9. PEDF treatment does not significantly affect mSOD or GPX-3 expression in LNCaP cells in an in vitro obesity setting..................40
Figure 10. PEDF treatment has no effect PC-3 cell viability or ROS/RNS....................................................................................42
Figure 11. PEDF treatment does not significantly affect mSOD or GPX-3 expression in PC-3 cells in an in vitro obesity setting..................43
Figure 12. PEDF treatment has no effect DU145 cell viability or ROS/RNS....................................................................................45
Figure 13. PEDF treatment does not significantly affect mSOD or GPX-3 expression in DU145 cells in an in vitro obesity setting..................46
LIST OF TABLES

Table 1. *In vitro* treatment groups...........................................................................24
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>ADT</td>
<td>androgen deprivation therapy</td>
</tr>
<tr>
<td>AHB</td>
<td>hydroxybutyrate</td>
</tr>
<tr>
<td>AKT</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>AP</td>
<td>anterior prostate</td>
</tr>
<tr>
<td>ATGL</td>
<td>adipose triglyceride lipase</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>BPH</td>
<td>benign prostatic hyperplasia</td>
</tr>
<tr>
<td>BRCA</td>
<td>breast cancer</td>
</tr>
<tr>
<td>CGS</td>
<td>cysteine-glutathione disulfide</td>
</tr>
<tr>
<td>CRPC</td>
<td>castration-refractory PCa cells</td>
</tr>
<tr>
<td>DCFH-DiOxyQ</td>
<td>DiOxyQ fluorogenic probe.</td>
</tr>
<tr>
<td>DLP</td>
<td>dorsolateral prostate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle medium</td>
</tr>
<tr>
<td>DRE</td>
<td>digital rectal exam</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>GPx</td>
<td>glutathione peroxidase</td>
</tr>
<tr>
<td>GSSG</td>
<td>oxidized glutathione</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>KO</td>
<td>knockout</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MITF</td>
<td>microphthalmia-associated transcription factor</td>
</tr>
<tr>
<td>OA</td>
<td>oleic acid</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCa</td>
<td>prostate cancer</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet derived growth factor</td>
</tr>
<tr>
<td>PEDF</td>
<td>pigment epithelium derived factor</td>
</tr>
<tr>
<td>PI3-K</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethanesulfonyle fluoride solution</td>
</tr>
<tr>
<td>PPA</td>
<td>periprosthetic adipose</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
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</tr>
<tr>
<td>PSA</td>
<td>prostate specific antigen</td>
</tr>
<tr>
<td>RNS</td>
<td>reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>SQA</td>
<td>subcutaneous adipose</td>
</tr>
<tr>
<td>TBS</td>
<td>tris-buffered saline</td>
</tr>
<tr>
<td>TBS-T</td>
<td>tris-buffered saline + tween</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor α</td>
</tr>
<tr>
<td>TNM</td>
<td>tumor node metastasis</td>
</tr>
<tr>
<td>TRAMP</td>
<td>transgenic adenocarcinoma of mouse prostate</td>
</tr>
<tr>
<td>TSP</td>
<td>thrombospondin</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VFP</td>
<td>visceral fat pad</td>
</tr>
<tr>
<td>VP</td>
<td>ventral prostate</td>
</tr>
<tr>
<td>WT</td>
<td>wildtype</td>
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“Somewhere, something incredible is waiting to be known.”

-Carl Sagan
Introduction:

Prostate Cancer

Prostate cancer (PCa) is the second most prevalent form of cancer in men in the United States. In 2013, there were over 238,000 new cases of PCa in American males (1). It is also a leading cause of cancer-related death, second only to lung cancer. A male in the United States has a 16.2% chance of developing PCa over his lifetime and a 12.5% chance of dying from it (2). Although frequently asymptomatic, the primary symptoms experienced as a result of PCa include difficult, painful, or frequent urination, a struggle to completely empty the bladder, any blood in the seminal fluid or urine, pain while ejaculating, or persistent pain in the back, waist, or pelvis (1).

The prostate is an important gland in the male reproductive system. It is located inferior to the urinary bladder, directly adjacent to the rectum, and it is responsible for the production and secretion of seminal fluid, which functions in the protection and motility of the sperm (3). The prostate is comprised of four functioning zones: the peripheral zone, the central zone, the transitional zone, and the anterior fibromuscuar zone (4). All zones, with the exception of the fibromuscuar zone, are glandular in structure (4). Over 95% of PCa cases are adenocarcinomas, which are derived from the epithelial cells within these glandular structures (5). Of these adenocarcinomas, 68% originate in the peripheral zone, 24% in the
transition zone, and 8% in the central zone (6). Prostates typically weigh 14-26 grams and do not usually continue to grow once a male reaches adulthood. Adenocarcinomas typically occur in a region of the prostate that is also susceptible to benign prostatic hyperplasia (BPH) (6). BPH is a post-adolescent enlargement of the prostate, which may cause the prostate to reach up to 49 grams (7). Although BPH has many similarities to PCa, including androgen-dependency and age-associated risk, its presence does not increase the risk of PCa in humans (8).

**PCa Diagnosis and Treatment**

Diagnosing PCa can be challenging for several reasons. One of the primary obstacles is that diagnosis involves a digital rectal exam (DRE) and a blood test for a prostate specific antigen (PSA) value, both of which may lead to unwillingness to participate by the patient, contributing to greater difficulty generating an accurate diagnosis (9). Additionally, the DRE is only 52-61% accurate (10), and the PSA antigen blood test lacks the high specificity desired in a diagnostic exam. Catalona *et al.* showed that of men with a serum PSA value ≥4.0 μg per liter, as values of <4.0 μg per liter are considered normal, only 22% actually had PCa (11). If elevated PSA is detected and if the DRE findings are abnormal, a transrectal ultrasound imaging technique is used to guide the collection of needle biopsy samples for histological evaluation (9). It is only with this histological evaluation that a firm diagnosis of PCa can be made.
A common scale for assessing prostate tumors at the time of biopsy is the Gleason score tumor grading system, which is based solely on the histologic appearance of the tumor cells and glandular structures. The tumor cells and glandular structures are graded on a scale of 1 to 5, based on their histological pattern, with 1 being benign and 5 being a highly aggressive cancer. As cancer in the prostate is typically heterogeneous, the two most common grades are summed to produce a final score. If the final score is less than 6, the tumor is considered to be well-differentiated and clinically non-cancerous. If a score is equal to 6, the tumor is considered to be moderately-differentiated and cancerous, although it is not considered an aggressive tumor. If the score is 7-10, the tumor is considered poorly-differentiated, cancerous, and aggressive (12).

Once the biopsy is identified as positive for PCa, the tumor can then be staged by its histological characteristics, tumor size, and primary or metastatic locations using the tumor, node, metastasis (TNM) system (13). When staging cancer, it is especially important to note whether the tumor has reached the regional lymph nodes, as this indicates that the tumor is able to spread to other locations throughout the body (14).

A conservative management approach is generally used for men with low grade tumors (a Gleason score of 6). To minimize the side effects from other treatment options, this approach is generally taken when a patient already has a life expectancy of less than 10 years (15). Conservative management is comprised of androgen deprivation therapy
Androgens are sex hormones that are normally responsible for the general maintenance and function of the prostate in healthy individuals. However, in high amounts, they have been shown to contribute to the pathogenesis of cancerous prostate cells (17). ADT has been shown to increase patient survival rates by blocking the androgen activity that promotes tumor growth (16). ADT can cause severe adverse effects, such as hot flashes, gynecomastia, and the flare phenomenon, which is a dangerous rise in serum testosterone levels (18). Due to these risks, an active surveillance approach has recently gained favor over other treatment options for low-grade cancers, particularly for men older than 70 (19). During this approach, no treatment is administered, and monitoring tests are periodically administered to assess the growth rate of the cancer (19).

If the tumor has remained localized to the prostate, surgery is also an option. A radical prostatectomy is the complete removal of the prostate gland and usually some of the surrounding tissue. It has been shown to significantly decrease PCa related mortality rates (20). However, the side effects of surgery may be more severe, including erectile dysfunction and urinary leakage (21). As with most cancers, chemotherapy and radiation therapy are also treatment options available to PCa patients.
PCa Risk Factors

The main risk factors for PCa are age, diet, and obesity, although race and family history are also important contributors (22). As men grow older, particularly over 50 years of age, they become far more susceptible to PCa due to the higher risk of accumulating enough sporadic genetic mutations in the prostatic epithelial cells to advance the cells to a cancerous phenotype (9). Some of these mutations may lead to the activation of oncogenes, which stimulate tumor growth, or to the inactivation or down-regulation of tumor suppressor genes, which prevent tumor growth (23). Hanahan and Weinberg have previously identified the six definitive characteristics tumor cells must acquire through genetic mutations to support their malignancy: 1) the ability to evade apoptosis, 2) the capability to metastasize and invade surrounding tissues, 3) the capacity to replicate limitlessly, 4) the capacity to sustain angiogenesis, the growth of new blood vessels from the existing vasculature, 5) the ability to maintain self-sufficiency in growth signal production, and 6) the ability to evade anti-growth signals (24). After their initial publication, they expanded these hallmarks to include 7) the deregulation of cellular energetics, 8) the avoidance of destruction by the immune system, 9) tumor-promoting inflammation, and 10) increased genomic instability and, thus, increased mutation (25). In addition to sporadic mutations, some individuals are carriers of tumor suppressor gene loss of function alleles that predispose them to developing PCa. Such loss of function alleles
occur in tumor suppressor genes such as breast cancer 1 (BRCA1), breast cancer 2, ataxia telangiectasia, checkpoint kinase 2, and BRCA1-interacting protein 1 (26). These mutations can serve as cancer biomarkers and aid in screening and tumor detection (9).

The clinical incidence rate of PCa is highest in African American men, due, in part, to several polymorphisms and androgen receptor mutations found in the African American population (27). African American men also have higher rates of obesity, which may contribute to their elevated PCa incidence rates (28). PCa incidence is significantly lower in men in certain Asian countries, including China and Japan (29). However, the incidence rate of PCa increases once a Chinese or Japanese man immigrates to the United States (30). These data collectively suggest that the differing clinical incidence rates between men from these regions and men who immigrated to the United States are due to factors other than genetic differences. Instead, environmental factors, such as altered diets or decreased exercise regimens, may explain these differences.

The transition to a Western diet, which generally includes an increased consumption of animal products high in fat and cholesterol and a decreased consumption of fruits and vegetables, is suspected to play a role. Such a change in diet would lead to increased lipid levels and weight gain, which could contribute to the increased clinical PCa incidence rates (31). This type of diet may also lead to a decreased intake of vitamins and minerals (32). Increased serum levels of certain vitamins, such as vitamin
D, are associated with decreased clinical incidence rates of PCa (33), indicating a role in the prevention of tumor cell proliferation.

Cholesterol, which is a steroid found in the cell membrane of animal cells, is also consumed in high amounts in an animal-based Western diet. Moreover, it has been shown to accumulate in the prostate during tumor formation, and the ability of prostate cells to maintain an appropriate balance of cholesterol is altered with increased age (34). Conversely, a study of New York men found that diets high in phytochemicals, which are naturally occurring chemicals in plants such as β-sitosterol, campesterol, stigmasterol, and phytosterols, are correlated with lower PCa incidence rates (35). Diets high in these phytochemicals are also generally low in fat and cholesterol (36). In addition, diets with high vegetable consumption, especially cruciferous vegetables, have been correlated with decreased PCa incidence rates (37).

**Obesity and PCa**

While there are conflicting studies as to whether or not obesity increases PCa incidence, the majority of studies show a clear association with more aggressive prostate tumors (38). The U.S. Centers for Disease Control and Prevention defines an obese individual as someone with a body mass index (BMI) of 30 or higher (39). The BMI measurement is calculated by a formula using a person’s weight and height (kg/m²) (39). Obese individuals are more likely to die once a prostate tumor has formed
(40), and obesity has been associated with higher recurrence rates of PCa after remission (41). Studies using a murine PCa model support these observations. Llaverias et al. conducted a dietary study using transgenic adenocarcinoma of mouse prostate (TRAMP) mice, which have a high PCa occurrence rate due to the insertion of the SV40 T antigen gene. After 20 weeks on a Western diet (21.2% fat and 0.2% cholesterol by weight), they found that prostatic tumors had increased microvascular density compared to TRAMP mice fed a control diet (0.5% fat and 0.002% cholesterol by weight) (31), indicating that the high fat diet was promoting angiogenesis, a well-established hallmark of cancer (44). The TRAMP mice fed a Western diet also had increased tumor incidence, enlarged prostates, and higher numbers of lung metastases compared to the controls (31).

While the above studies strongly support a role for diet in PCa progression, the mechanism by which consumption of a high fat diet and obesity specifically contributes to PCa progression is unclear. Increased adipose depots may promote the progression of PCa through a variety of molecular mechanisms, including the increased secretion of pro-angiogenic factors, growth factors or cytokines, sex hormones, and fatty acids. The enlarged adipose tissue may also lead to a chronic inflammatory state that could promote tumor growth. Obesity-stimulated PCa progression is likely due to a combination of these contributing factors (42).
More specifically, obesity may contribute to metabolic syndrome, a group of metabolic abnormalities such as glucose intolerance and dyslipidemia, the presence of which positively correlates with the presence of PCa (43). The altered levels of certain adipose-derived cytokines, also known as adipokines, may also be a link between obesity and aggressive PCa. When treated with obese human periprostatic adipose tissue secretions taken from lean and obese PCa patients, both PCa and normal prostate epithelial cells demonstrated an increase in cellular proliferation (44). Additionally, the unsaturated to saturated fat ratio from these tissues was negatively correlated with the histological grade of the tumor (44). Certain adipokines, such as leptin, are thought to promote PCa progression. In an in vitro study, Hoda and Popken showed that leptin treatment promoted PCa cellular proliferation and decreased the number of apoptotic PCa cells via the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3-K) signaling pathways (45). In addition to an increase in cellular proliferation in vitro, Moreira et al. also showed that treatment of RM1 PCa cells with 3T3-L1 mature adipocyte cell conditioned media led to an increase in invasion and migration (46). Furthermore, Noda et al. were able to obtain similar results after treating PCa cells with leptin for 28 days, and they also found that leptin treatment increased the expression of the leptin receptor, ObR, in the PCa cells (47). Conversely, decreased plasma levels of a different adipokine, adiponectin, have been linked to increased PCa incidence, tumor grade, and disease
stage (48). Tan et al. showed that adiponectin is down-regulated in PCa tissues compared to those taken from BPH patients (49). They also showed that silencing exogenous adiponectin in 22RV1 human PCa cells further promoted tumor cell proliferation and initiated the epithelial to mesenchymal transition process, contributing to their invasive potential (49).

Another theory regarding the link between obesity and aggressive PCa is the contribution of a chronic inflammatory state caused by increased adipose tissue. Obesity leads to an increase in the release of fatty acids and pro-inflammatory molecules. Weisberg et al. found that pro-inflammatory macrophages accumulate in obese adipose tissue and contribute to the secretion of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), further promoting a pro-inflammatory state (50). Subsequently, Park et al. demonstrated that obesity-fueled hepatocellular carcinomas are dependent on the increased production of TNF-α and IL-6, which lead to the activation of an oncogenic transcription factor, STAT3, further supporting the role of an inflammatory state in obesity-induced PCa progression (51).

One final theory regarding the mechanism linking obesity and aggressive PCa is that lipid overload in both adipose and non-adipose cells causes altered cellular metabolism. This may be an effect of a high fat diet, obesity, and/or increased age and accumulated genetic mutations, which are characteristics in cancer cells. Cancer cells are known to have
altered metabolism, with one example being the increased amount of glucose uptake and lactate production of cancer cells, a metabolic change known as aerobic glycolysis or the Warburg effect (52). These alterations in cellular metabolism may lead to the increased generation of by-products in the mitochondria that contribute to increased oxidative stress within the tissue (53), further promoting PCa progression.

**Pigment Epithelium Derived Factor**

First identified as a secreted protein in retinal cells by Joyce Tombran-Tink and Lincoln Johnson in the 1980s, pigment epithelium-derived factor (PEDF), a 50 kDa glycoprotein, is a non-inhibitory member of the serpin family proteins. In the eye, PEDF is present in photoreceptors and inner retinal cells, although it may be secreted into the interphotoreceptor matrix (54). Tombran-Tink et al. have identified PEDF in a variety of tissues, including the liver, testis, ovaries, pancreas, and brain (55). The full crystal structure of glycosylated PEDF is 2.85 angstroms and has an asymmetric charge distribution (56). PEDF has a variety of functions, including the regulation of cell migration, proliferation, the inhibition of vasculature development, the regulation of cancer cell apoptosis, and the promotion of retinal neuron differentiation. PEDF has two functional epitope regions, a 34-mer peptide and a 44-mer peptide (57). The 44-mer peptide can interact with receptors to induce a neurotrophic function, protecting and aiding in the development of neurons
(58), and the 34-mer peptide induces apoptosis and blocks the migration of endothelial cells (59).

Compared to benign prostate tissue, PEDF expression is decreased or absent in human PCa tissues (60). In Dunning rats, more aggressive metastatic PCa tumors also have decreased PEDF expression compared to benign tissues, suggesting that loss of PEDF may promote a metastatic phenotype (61). Additionally, PEDF is a potent anti-angiogenic factor; it inhibits angiogenesis, the growth of new blood vessels from the existing vasculature, via the binding of multiple integrins on endothelial cells, although the exact mechanisms remain unclear (62). This angiogenic process is regulated by a number of factors that either inhibit or promote angiogenesis. Pro-angiogenic factors include interleukin-8, fibroblast growth factor (FGF-2), vascular endothelial growth factor (VEGF), and platelet derived growth factor (PDGF) (63), while angiogenesis inhibitors include thrombospondin-1 (TSP-1) (64) and interleukin-12 (IL-12) (65). Famulla et al. have shown that during adipogenesis in human primary adipocytes and smooth muscle cells, the expression of PEDF significantly increases, while TNF-α and hypoxic conditions both contribute to the down-regulation of PEDF (66). However, in contrast, Yang et al. found that hypoxic conditions cause an increase in the expression of PEDF and VEGF in retinal glial Müller cells (67). In a mouse model, lack of PEDF promoted the development of stromal vascularity and epithelial cell hyperplasia in the prostate (60). These data
cumulatively suggest that PEDF may potentially have therapeutic use for cancer patients by inhibiting blood vessel growth (68).

In murine studies, *in vivo* PEDF has demonstrated the ability to suppress neovascularization in the eye (69). Regulation of blood vessel growth in the eye is necessary for proper visual function. This regulatory function was also shown in an *in vitro* setting, along with the ability to inhibit VEGF-promoted retinal endothelial proliferation, suggesting PEDF treatment as a potentially effective treatment for retinal neovascular diseases (70). PEDF has also demonstrated the ability to prevent light-induced photoreceptor cell death via the activation of the Akt signaling pathway when introduced *in vitro* (71). Additionally, Spranger *et al.* analyzed the expression of PEDF in patients with and without hypoxic eye diseases, including proliferative diabetic retinopathy and extensive nondiabetic retinal neovascularization, and found that PEDF is underexpressed in individuals with these diseases (72). They also proposed that these patients may benefit from treatment with angiogenesis inhibitors, including PEDF (72). PEDF has been described as having a high therapeutic potential due to its ability to target new blood vessel growth, easy administration as a soluble protein or via viral-mediated gene transfer, and due to its high stability and efficacy (73). In a phase I clinical trial, patients with advanced macular degeneration were given intravenous injections of an adenoviral vector expressing PEDF. PEDF treatment produced an anti-angiogenic effect in these patients, with 94% and 71%
experiencing a decrease or no increase, respectively, in lesion size at 3 and 6 months (74).

**PEDF’s Role in Tumor Progression**

PEDF expression is decreased in a variety of tumor types. In human breast cancer tissue, PEDF expression is significantly decreased compared to benign breast tissue (75). In melanoma cells, PEDF expression positively correlates with microphthalmia-associated transcription factor (MITF) expression, which is characteristic of less-aggressive tumors (76). The silencing of MITF also down-regulates PEDF expression in these cells, implicating a causal relationship (76). In breast cancer, PEDF inhibited cancer cell migration and invasion via the down-regulation of fibronectin and MMP2/MMP9 by utilizing the p-ERK and p-AKT signaling pathways (77). An *in vivo* study with mouse neuroblastomas showed that Schwann and ganglionic cells produce PEDF, but more poorly-differentiated tumor cells do not; additionally, recombinant PEDF was shown to have antitumor function in neuroblastomas both *in vitro* and *in vivo* (78).

The expression of PEDF is also down-regulated in human PCa tissues and in PCa cell lines (DU145, LNCaP, and PC-3) compared to benign human tissues and normal prostate epithelial cells (60). Treatment of xenograft PCa tumors from the PC-3 and DU145 cell lines with PEDF targets angiogenesis and initiates apoptosis, supporting PEDF as a
treatment to suppress tumor growth (60). In vitro, increased expression of PEDF inhibits the migratory ability of castration-refractory PCa (CRPC) PC-3 cells. CRPC is an aggressive form of PCa currently without viable treatment options. In vivo, PEDF decreased the growth of subcutaneous LNCaP, PC-3, and CL1 xenograft tumors (79). Nelius et al. showed that PEDF treatment, in conjunction with low-dose chemotherapy, significantly increased the survival time of mice with LNCaP-derived CRPC xenograft tumors (79).

In the PC-3 prostate cancer cell line, the overexpression of PEDF via an adenoviral vector caused a reduction in cell proliferation (80). Microarray analysis showed that PEDF regulates genes responsible for angiogenesis, signal transduction, cell growth, and apoptosis (80). Administration of PEDF to DU145 and PC-3 PCa cells has also been shown to alter expression of genes controlling catalytic activity (17β-hydroxysteroid dehydrogenases), cell proliferation (fibroblast growth factor 3), angiogenesis (brain-specific angiogenesis inhibitor 2), and apoptosis (growth arrest and DNA-damage-inducible protein alpha) (81). When co-cultured with PEDF and adipose-derived mesenchymal stromal cell conditioned media, PC-3 cells exhibited down-regulation of a metastatic gene (pescadillo homolog), androgen-encoding genes (androgen receptor, sex hormone-binding globulin), genes regulating the phosphoinositide 3-kinase-protein kinase B (AKT) signaling pathway (AKT1, B-cell lymphoma 2, S-specific cyclin-D2), an inflammatory gene (cytochrome C oxidase
subunit 3), and a survival gene (insulin-like growth factor 1), reducing the invasiveness and growth potential of the PC-3 cells (82).

PEDF knockout (KO) C57BL6 mice, which do not express the PEDF protein, develop prostate hyperplasia, a PCa precursor in mice, by three months of age (60). Doll et al. characterized this development via increased cellular proliferation and microvessel density (60). PEDF KO mice also have elevated intracellular lipid accumulation in both adipose and non-adipose tissues, including the prostate (83). Additionally, PEDF KO mice exhibit increased visceral adipose tissue surrounding the prostate (Doll et al., unpublished observations) and have 50% increased total body fat compared to controls (84). PEDF deficient mice also develop hepatic steatosis, also referred to as fatty liver disease (83).

**PEDF and Metabolism**

PEDF is also believed to play an important role in lipid metabolism. Elevated lipid levels, like those occurring with a high fat diet and ectopic lipid accumulation, cause cellular dysfunction and death in many normal tissue types (85). In murine skeletal muscles, PEDF has been shown to induce the metabolism of fatty acids via the promotion of lipolysis (86), a mechanism dependent on adipose triglyceride lipase (ATGL) (87). Db/db obese mice, which are null for the leptin receptor (88), are known to develop hepatic steatosis (89). In one study, the authors observed decreased PEDF levels in the liver of db/db mice compared to wildtype
C57BL/6J liver tissues (89). Together, these data suggest that PEDF regulates lipid deposits in the liver. Chung et al. also showed that PEDF regulates lipid metabolism in hepatocytes treated with recombinant PEDF (83). However, cancer cells have altered metabolisms, and how PCa cells react to lipid overload and the role of PEDF in this process remains unknown.

In one study using C57BL/6J mice fed a high fat diet (60% calories from fat) for 16 weeks, the average plasma PEDF concentration, 4.9 ± 1.6 ng/ml, was increased 3.2-fold as compared to mice fed a control diet (86). In a given patient population, elevated serum PEDF levels positively correlate with the presence of metabolic syndrome (90). In human adipocytes, secretion of PEDF is also increased by insulin, a hormone secreted to aid in the regulation of lipid metabolism (66). Additionally, in Japanese patients, elevated serum levels of PEDF have been positively associated with the metabolic features of type 2 diabetes, including elevated levels of triglycerides, TNF-α, and creatinine (91). While these data implicate PEDF in disease processes, it is still unclear if PEDF plays a causative role or if it is functioning as a counter-balance system in glucose and/or lipid metabolism, as proposed by Gattu et al (92).

**PEDF and Oxidative Stress**

Ectopic lipid accumulation, such as occurs in an obese setting, can cause increased oxidative stress in cells. Cellular damage due to oxidative
stress results from increased levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS). These molecules are highly reactive oxygen-containing and nitrogen-containing molecules, respectively, and they are the by-products of normal cellular reactions, primarily in the mitochondria. Low levels of ROS/RNS molecules are crucial for multiple biochemical processes, including those for cellular differentiation (93), apoptosis (94), and immunity (95). Some examples of ROS include the hydroxyl radical, nitric oxide, and the superoxide anion, while examples of RNS include nitrogen dioxide and peroxynitrite. The levels of ROS and RNS are tightly regulated by antioxidants, such as glutathione peroxidase (GPx) and superoxide dismutase (mSOD), due to the potential cellular damage that these molecules can inflict (96). GPx functions to prevent the lipid peroxidation of cellular membranes by reducing free hydrogen peroxide to water, while mSOD catalyzes the conversion of superoxide to hydrogen peroxide, which is then reduced to water by GPx or other antioxidants (97). When ROS/RNS levels rise above the antioxidant capacity, cellular damage occurs. Decreasing lipid levels can reduce oxidative stress. For example, decreasing dietary lipid consumption has been shown to reduce ROS and other oxidative stress markers in the endothelial cells of rabbits (98). Damaged molecules, such as proteins and lipids, must then be degraded and replaced. Damage to cellular nucleic acids, such as DNA and RNA, can result in mutations that, in turn, may promote tumor formation and progression.
In PCa cells, ROS levels are increased as compared to normal WPMY1 and RWPE1 prostate epithelial cell lines (99). Higher ROS levels have also been found in more aggressive PCa cell lines, such as PC-3 (99). In humans, PCa and prostatic intraepithelial neoplasia tissues also have decreased levels of certain antioxidant enzymes, including SOD1, mSOD, and catalase, further implicating changes in oxidative stress in tumor development and progression (100). Additionally, sera metabolic data reveal changes in circulating markers of oxidative stress in PEDF KO mice as compared to wildtype (WT) C57BL/6J mice. WT mice fed a high fat diet (32.2% kcal from fat) had elevated levels of oxidized glutathione (GSSG) and cysteine-glutathione disulfide (CGS) and decreased levels of 2-hydroxybutyrate (AHB) (Doll et al., unpublished observations), reflective of a normal response to the oxidative stress induced by a high fat diet (101). In PEDF KO mice fed the same high fat diet, AHB levels significantly increased, but GSSG and CGS remained unchanged compared to PEDF KO fed a control diet. On the control diet, PEDF KO mice had significantly decreased levels of AHB compared to the WT on a control diet (Doll et al. unpublished observations). As discussed above, PEDF KO mice develop a prostatic intraepithelial neoplasia-like hyperplasia phenotype, a precursor lesion to cancer. Then the PEDF KO mice were fed a high fat diet, the precursor phenotype was pushed to cancerous phenotype (Doll et al. unpublished observations).
Additionally, PEDF has demonstrated antioxidant activity by decreasing H$_2$O$_2$-induced apoptosis in granuloma cells (102), although another study on granuloma cells suggested that PEDF treatment induced ROS production in a concentration-dependent manner (10-100nM) (103). PEDF treatment also blocked angiotensin II-induced ROS generation in human lymphoblastic leukemia MOLT-3 T cells (104). These data cumulatively suggest that PEDF loss interferes with the normal cellular response to oxidative stress.

**Summary and Gap in Knowledge**

The presence of aggressive PCa is positively correlated with obesity and consumption of a high fat diet (38). PEDF, an angiogenesis inhibitor, has been shown to regulate lipid metabolism and have antioxidant properties in normal cell types (89) and may play a key role in lipid regulation in the prostate. Additionally, PEDF has demonstrated antioxidant activity in some other cell types (102). While PEDF function as an angiogenesis inhibitor in the prostate has been well established (60), its function in lipid regulation and as an antioxidant have not been established in the prostate. Although ROS has been linked to increased tumor progression (99), PEDF’s antioxidant ability has not been explored in the prostate, adipose tissue, or in PCa cells. Here, studies were conducted to begin to elucidate the molecular pathway of PEDF-mediated regulation of lipid metabolism and to establish a potential antioxidative role.
of PEDF in prostate tissue, adipose tissue, and PCa cells. Toward these studies, two specific aims were addressed.

**Hypothesis and Specific Aims:**

*Central Hypothesis:* PEDF demonstrates antioxidant activity in the prostate and adipose tissue by reducing ROS/RNS and increasing antioxidant enzyme levels.

**Specific Aim 1:** Quantify oxidative stress levels in prostate and adipose tissues taken from wildtype C57BL/6J and PEDF KO mice.  
*Working hypothesis:* Loss of PEDF will lead to increased ROS/RNS levels and decreased antioxidant enzyme levels in both prostate and adipose tissues.

**Specific Aim 2:** Determine the effect of PEDF on lipid accumulation-induced changes to oxidative stress levels in prostate and PCa cells.  
*Working hypothesis:* Lipid accumulation will increase ROS/RNS and decrease antioxidant enzyme levels in prostate and PCa cells, while the addition of PEDF will decrease ROS/RNS and increase antioxidant enzyme levels.
Materials and Methods:

Prostate and Adipose Tissue Collection and Explant Culture

The ventral (VP), anterior (AP), and dorsolateral (DLP) lobes of the prostate and the subcutaneous (SQA), visceral fat pad (VFP), and periprostatic (PPA) adipose tissues were dissected from 4-month-old wild type and PEDF KO mice. All mice were of C57BL/6J background. The mice were anesthetized with isoflurane and euthanized by cervical dislocation. The tissues were exposed via a midline incision through the abdominal wall. The SQA tissue was excised, and an incision was made through the peritoneum. The VFP was harvested, and then the PPA tissue and VP, DLP, and AP prostate lobes were excised under a dissecting microscope. All tissues were immediately frozen in liquid nitrogen for later use in oxidative stress assays or placed in a tube on ice for explant culture.

For the explant culture, the weight of each tissue was recorded, and the tissue was washed twice with PBS in a culture dish. The tissues were cut and weighed, then plated with serum-free (basal) Dulbecco’s Modified Eagle Medium (DMEM) a concentration of 25 μg/mL. The explant cultures were treated with 10 μM H2O2 or an equal volume of PBS and incubated at 37°C for 3 hours. After incubation, the tissues were collected, washed with PBS, and stored at -80°C until use in the oxidative stress assay.
**Cell lines and culture conditions**

RWPE-1, DU145, PC-3, and LNCaP cell lines were purchased from American Type Culture Collection Company (Manassas, Virginia). RWPE-1 cells were originally isolated from normal human prostatic epithelial cells and transfected with human papillomavirus 18 to immortalize them (105). DU145 cells were originally isolated from a brain metastasis of a human prostate adenocarcinoma (106). PC-3 cells were isolated from a human prostate adenocarcinoma that metastasized to bone (107). LNCaP cells were isolated from a human prostatic carcinoma that metastasized to a regional lymph node (108). Cells were maintained in growth media [Keratinocyte growth media for RWPE-1; DMEM with 10% FBS and 1% P/S for DU145 and PC-3, and Roswell Park Memorial Institute (RPMI) media for LNCaP] at 37°C with 5% CO₂.

**In vitro treatment with PEDF, Oleic Acid, and H₂O₂**

Cells were plated at 25,000 cells/cm² on 10 cm tissue culture dishes and incubated at 37°C, 5% CO₂ overnight. After incubation, the growth medium was gently aspirated, and the cells were washed with sterile PBS. Basal media was added to each plate, and the cells were returned to the incubator for 4 hours. The basal media was gently aspirated, and new basal media was added with the treatments outlined in Table 1. Group 1, with only basal media, served as the negative control.
The cells were incubated with oleic acid (Sigma-Aldrich, Saint Louis, MO) and PEDF (Sigma-Aldrich, Saint Louis, MO) only for 45 hours at 37°C, 5% CO₂, then H₂O₂ was added for the final 3 hours. OA was chosen to mimic an obesity setting *in vitro*, as it has been observed to promote the proliferation of PCa cells and inhibit PEDF expression (Doll lab, unpublished observations). After incubation, the conditioned media and the cell lysates were collected as described below.

### Table 1: *In vitro* treatment groups

<table>
<thead>
<tr>
<th>Group</th>
<th>PEDF (50 nm)</th>
<th>OA (1 mM)</th>
<th>H₂O₂ (10 μM)</th>
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#### Cell lysate and conditioned media collection

The conditioned media (CM) was collected and placed on ice. The CM was then centrifuged at 2500 x g for 10 minutes to pellet any cellular debris and transferred to a new tube. 1X protease inhibitor cocktail (Sigma-Aldrich, Saint Louis MO) and 100 nM phenylmethanesulfonyl fluoride solution (PMSF, Sigma-Aldrich, Saint Louis MO) was added to a final dilution of 1:100. The cells remaining on the plate were washed with PBS and trypsinized. 4 mL of growth media was added to stop
trypsinization, and the cells and media were collected into a conical vial. A 50 μL aliquot of the cell solution was mixed with 50 μL of 0.4% trypan blue solution (Sigma-Aldrich, Saint Louis, MO) to provide a cell count and permit the assessment of cell viability using a Cellometer (Nexcelom, Lawrence, MA). 20 μL of the cell mixture was placed in each chamber of a Cellometer counting slide. The cell aliquots were counted in duplicate on the Cellometer, per manufacturer’s instructions. The total cell number, live cell number, and cell viability were recorded. The remaining cells were pelleted by centrifugation at 800 x g for 8 minutes, and the supernatant was gently aspirated. The cell pellets and conditioned media were stored at -80°C until needed.

**Oxidative Stress Assay**

Oxidative stress levels were assessed using the Oxiselect STA-347 ROS/RNS Assay Kit from Cell Biolabs, Inc. (San Diego, CA), which quantifies the total amount of ROS/RNS in a tissue or cell lysate sample, using a dichlorodihydrofluorescin DiOxyQ (DCFH-DiOxyQ) fluorogenic probe. The primary tissues, explant culture tissues, cell lysates, and conditioned media were assayed. Cell pellets were resuspended in sterile PBS at a concentration of 1 x 10^6 cells/mL. Cells were sonicated at 40% amplitude for 30 seconds in duplicate. Tissues were homogenized in sterile PBS at a concentration of 25 mg/mL using a Kontes motor and disposable pestles (Fisher Scientific, Waltham, MA). A standard curve was
created using $\text{H}_2\text{O}_2$. Samples and standards were loaded onto a 96-well plate. A catalyst was added to each well as the DiOxyQ probe was primed and stabilized in solution per the manufacturer’s instructions. The probe was added to each well and incubated, protected from light, for 30 minutes. The plate was then read at the excitation and emission wavelengths of 480 nm and 530 nm, respectively, using a Synergy HT plate reader (Biotek, Winooski, VT) and Gen5 software.

**Assessment of GPx-3 and mSOD Levels**

The total amount of protein in each cell lysate and tissue homogenate sample was quantified using a Coomassie dye-binding assay. 490 μL of Coomassie dye was aliquoted into microfuge tubes, and 5 μL of each sample was added. Samples were incubated for 10 minutes, and then transferred to a 96 well plate, along with albumin standards in PBS. The standards had final concentrations of 0 μg/mL, 25 μg/mL, 125 μg/mL, 250 μg/mL, 500 μg/mL, 750 μg/mL, 1000 μg/mL, and 1500 μg/mL. The plate was read at 595 nm using the Synergy HT plate reader. Cell pellets were resuspended in 1 mL sterile PBS and sonicated at 40% amplitude for 30 seconds in duplicate. Tissues were homogenized in tissue extraction reagent (Invitrogen, Carlsbad, CA) at a concentration of 10 mg/mL using a Kontes motor and disposable pestles (Fisher Scientific, Waltham, MA). The volume of cell lysate and tissue homogenate needed
for 20 μg of protein was transferred to a new tube and lyophilized in 3-4 20 minute cycles each, with 15 minutes of medium-high heat.

A Western blot was then used to compare the amount of GPx3 and mSOD in the cell lysate of each treatment group, as the levels of these enzymes are indicators of oxidative stress within the cells. The proteins were separated using a sodium dodecyl sulfate (SDS)-polyacrylamide gel comprised of a 5% stacking gel and a 7.5% resolving gel in a 0.1% SDS running buffer. 20 μg of the total protein was placed in a microfuge tube with an equal volume of 2X Laemmeli sample loading buffer. The lysate samples were incubated at 100°C for 10 minutes to ensure denaturation of the proteins. 30 μL of each sample and 10 μL of a Precision Plus protein molecular weight standard (BioRad, Hercules, CA) was loaded onto the gel. The gel was run at 110 volts for 1.5 hours to separate the proteins based on size, with GPx-3 measuring 92 kDa, mSOD measuring 22 kDa, and GAPDH measuring 37 kDa.

Next, the proteins in the gel were transferred to a nitrocellulose membrane by electroblotting. Prior to setup, the membrane was soaked in methanol for 30 seconds, then rinsed 10 times with diH₂O. The gel and membrane were positioned between 4 pieces of Whatman filter paper and 2 sponges soaked in 1X Tris-glycine transfer buffer for 10 minutes prior to blotting. The transfer setup was placed in a transfer cassette, and the transfer was conducted at 70 volts for 2 hours on ice. The membrane was
washed with 1X tris-buffered saline (TBS) + 0.1% tween (TBS-T) for 5 minutes to remove any residual SDS.

To prepare for antibody hybridization, the membrane was blocked using TBS with 5% powdered milk to prevent any nonspecific binding by the antibody to the membrane. The membrane was then washed with TBS-T and incubated with the primary antibody at a 1:1000 dilution in TBS-T with 5% milk overnight at 4°C. Anti-GPx3, anti-mSOD, and anti-GAPDH (Cell Signaling, Danvers, MA) antibodies were the primary antibodies used for protein detection. GAPDH served as a control for total protein loading between samples. After washing 3 times for 10 minutes with TBS-T, a horseradish peroxidase-linked secondary antibody was used to bind to the primary antibody for 1 hour at room temperature. 3 final TBS-T washes were performed, and chemiluminescence was used to visualize the protein. Super Signal West Pico ELC substrates (Thermo Fisher Scientific, Milwaukee, WI) were mixed in a 1:1 ratio. The membrane was placed, protein-face down, into the solution for 1 minute. The membrane was then saran-wrapped and placed in a film cassette. In a dark room, several film exposures at various times were performed, and the film was developed with an automated TI-200 developer (Kodak, Rochester, NY). The exposure time was adjusted as needed to obtain an appropriate image for comparison between lanes. If no bands were visible after development, the membrane was re-washed with TBS-T and a more sensitive chemiluminescence detection system was used. Super Signal
West Femto ELC substrates (Thermo Fisher Scientific, Milwaukee, WI) were mixed in a 1:1 ratio, and the membrane was placed, protein-face down, into the solution for 1 minute. The membrane was placed in the cassette and exposed as described above.

To probe with a second primary antibody, the first antibody was stripped from the membrane. The membrane was washed with TBS-T for 5 minutes, then submerged in a boiling 0.1% SDS stripping buffer and allowed to cool to room temperature. It was washed thrice with TBS-T. The second primary antibody was hybridized, beginning with the blocking step as described above. The amount of GPx3 and mSOD was normalized to GAPDH as fold over values. Protein levels were compared between sample lanes, relative to untreated wild type tissues, by densitometry using ImageJ software.

Statistics

All in vitro experiments were repeated in duplicate. A Student’s t-test was used to analyze the results. For analyses where normality or equal variance was not reached, a Mann Whitney U test was used. For each tissue type, the ROS/RNS assay levels were compared between WT and PEDF KO groups. For each cell type, the levels of oxidative stress markers were compared between each treatment group. Oxidative stress markers were compared between the PCa cell lines and the normal prostate cell line for each treatment group. All analyses were performed by
statistical analysis software within the SigmaPlot program (v12.0, Systat Software, Inc, San Jose California). A result was considered statistically significant when P≤0.05.

Results:

Part I. In vivo and ex vivo studies

The effect of PEDF loss on oxidative stress in prostate and adipose tissues

The oxidative stress levels in prostate and adipose tissues were quantified by both an ROS/RNS assay (Oxiselect) and by comparison of GPx3 and mSOD levels. Of the three adipose tissue depots tested, the PPA and the VFP tissues from PEDF knockout mice had significantly increased ROS/RNS levels compared to wildtype tissues as a control (Figure 1A; n=4/group; p=0.0380 and p=0.0342, respectively). The ROS/RNS levels in the SQA tissue from PEDF KO mice were also increased; however, this did not reach statistical significance (Figure 1A; n=4/group; p=0.057). In the prostate tissues, while a trend toward increase in ROS/RNS levels was observed in the VP and AP tissues in the PEDF KO mice, this was not statistically significant (Figure 1B).
Figure 1. Loss of PEDF increases oxidative stress in adipose but not prostate tissues. Prostate and adipose tissues were collected from wildtype C57Bl/6J and PEDF KO mice (n=4/group). Tissues were homogenized, and RNS/RNS levels were quantified using an Oxiselect ROS/RNS assay in the (A) subcutaneous adipose (SQA), periprostatic adipose (PPA), visceral fat pad (VFP), and (B) ventral prostate (VP), dorsolateral prostate (DLP), and anterior prostate (AP) tissue homogenates. Levels from PEDF KO mice were normalized to wildtype. * p<0.05 compared to WT, # p = 0.057 compared to WT.

Loss of PEDF exhibits no significant change on GPX-3 and mSOD levels in adipose and prostate tissue

Homogenized adipose (SQA, PPA, and VFP) and prostate (VP, DLP, and AP) mouse tissues were assessed by Western blot for GPx-3 and mSOD levels. For both the adipose and prostate tissues, there were no statistically significant changes in mSOD or GPx-3 expression levels between PEDF KO and control wildtype tissues (Figure 2 and Figure 3, respectively). Within the adipose tissue depots, PEDF KO mice showed a trend toward decrease in mSOD expression in both VFP and PPA, although none of these differences reached statistical significance (Figure 2A). Similarly to mSOD, GPx-3 expression in the SQA, VFP, and PPA was also decreased in PEDF KO mouse tissues (Figure 2B). In the prostate...
tissues, no noticeable differences were seen between the PEDF KO and WT mSOD expression levels (Figure 3A), and only the DLP tissues demonstrated a change in enzyme expression with an increase in GPx-3 expression (Figure 3B).

Figure 2. PEDF loss exhibits no significant change on GPX-3 and mSOD levels in adipose tissue. Adipose tissues were collected from wildtype C57Bl/6J and PEDF KO mice (n=2/group). Tissues were homogenized, and GPx-3 and mSOD levels were assessed via Western blotting from subcutaneous adipose (SQA), periprostatic adipose (PPA), and visceral fat pad (VFP) tissue homogenates. Levels were normalized to GAPDH, and Image J software was used to assess relative (A) mSOD and (B) GPx-3 expression levels.

Figure 3. PEDF loss exhibits no significant change on GPX-3 and mSOD levels in prostate tissue. Prostate tissues were collected from wildtype C57Bl/6J and PEDF KO mice (n=2/group). Tissues were homogenized, and GPx-3 and mSOD levels were assessed via Western blotting from ventral prostate (VP), dorsolateral prostate (DLP), and anterior prostate (AP) tissue homogenates. Levels were normalized to GAPDH, and Image J software was used for densitometry, and relative (A) mSOD and (B) GPx-3 expression levels are presented. No values reached statistical significance.
Absence of PEDF does not alter response to H$_2$O$_2$ ex vivo in adipose or prostate tissues

To assess if absence of PEDF impairs the tissue response to an oxidative stress stimulus, in a pilot study, SQA, PPA, and VFP adipose tissues were treated with or without H$_2$O$_2$ for 3 hours ex vivo (n=2/group). An Oxiselect assay was performed on the tissues to measure the total ROS/RNS concentration in each tissue sample. No statistically significant differences were observed between the wildtype and PEDF KO adipose tissues (Figure 4). Surprisingly, the H$_2$O$_2$-treated tissues also exhibited no significant changes compared to the respective untreated tissues. Likewise, no statistically significant differences were observed between the wildtype and PEDF KO prostate tissues (Figure 5), nor did the tissues cultured with H$_2$O$_2$ exhibit any statistically significant change compared to the untreated tissues.

Figure 4. Loss of PEDF exhibits no significant change on oxidative stress in response to H$_2$O$_2$ stimulus in adipose tissue. Adipose tissues were collected from wildtype C57Bl/6J and PEDF KO mice (n=2/group). Tissues were treated with H$_2$O$_2$ or PBS, and then homogenized. RNS/RNS levels were assessed for the (A) subcutaneous adipose tissue, (B) periprostatic adipose tissue, and (C) visceral fat pad using an Oxiselect ROS/RNS assay. No values reached statistical significance.
Part II. *In vitro* obesity studies

Section A. RWPE-1

**PEDF treatment has no effect on RWPE-1 cell viability**

Normal prostatic epithelial cells, RWPE-1, were treated with PEDF in the presence and absence of OA. H$_2$O$_2$ was added to specified treatment groups to induce the production of ROS and as a positive control for the ROS/RNS assay. The effect of PEDF on cell viability was being measured with and without OA present to simulate an obese environment. A negative control group received only basal media in lieu of additional treatment. In the presence and absence of OA, PEDF had no significant effect on cell viability of RWPE-1 cells (Figure 6A). OA treatment alone also produced no change in cell viability, nor did H$_2$O$_2$.
treatment alone. Only the dual OA and H$_2$O$_2$ treated cells exhibited a significant decrease in cell viability compared to untreated (Figure 6A, p=0.00851).

**PEDF treatment does not affect oxidative stress in RWPE-1 cells**

An Oxiselect assay was performed on PEDF, OA, and H$_2$O$_2$ treated RWPE-1 cell lysate and conditioned media to measure the total ROS/RNS concentration in each sample. No statistically significant changes in ROS/RNS levels were observed in RWPE-1 cell lysate (Figure 6B) or conditioned media (Figure 6C). Interestingly, cells treated with H$_2$O$_2$ alone did not exhibit an increase in ROS/RNS levels. The expression levels of mSOD, GPx-3, and GAPDH were also assessed in the cell lysates of the treated RWPE-1 cells via Western blotting and antibody hybridization. For densitometry, mSOD (Figure 7A) and GPx-3 (Figure 7B) expression levels were normalized to GAPDH expression within each sample. These results show that treatment with PEDF, in the presence and absence of OA, did not significantly affect the expression of mSOD or GPx-3 compared to the untreated control group or the OA-treated group.
Figure 6. PEDF treatment has no effect on prostate or PCa cell viability or ROS/RNS. Normal prostate epithelial cells, RWPE-1, were treated with PEDF (50 nM), oleic acid (OA; 1 mM), and/or H$_2$O$_2$ (10 µM). PEDF and OA treatments were for 48 hours, with the H$_2$O$_2$ treatment for the last 3 hours of the treatment. Conditioned media was collected, then cells were trypsinized, an aliquot was taken for cell count, and cell lysate was collected. (A) Cell viability was assessed on a Cellometer, and ROS/RNS levels were quantified in the cell lysate (B) and conditioned media (C) using an Oxiselect assay. ** p<0.00002 compared to untreated cells. Results shown are the combined data of two independent experiments.
Section B. LNCaP

PEDF treatment has no effect on LNCaP cell viability

Androgen-dependent PCa cells, LNCaP, were treated with PEDF in the presence and absence of OA and H2O2. PEDF treatment alone did not affect the viability of LNCaP cells (Figure 8A). OA treatment alone significantly decreased cell viability compared to untreated (Figure 8A, p=0.0135). In the presence of both PEDF and H2O2, OA further decreased cell viability compared to the PEDF and H2O2-treated (Figure 8A, p=0.0195). Additionally, dual treatment with OA and H2O2 significantly decreased viability compared to untreated (Figure 8A, p=0.00308).
PEDF treatment does not affect oxidative stress pathways in LNCaP cells

An Oxiselect assay was performed on PEDF, OA, and H$_2$O$_2$ treated LNCaP cell lysate and conditioned media to measure the total amount of ROS/RNS in each sample. For the cell lysate, only the cells treated with PEDF, OA, and H$_2$O$_2$ demonstrated an increase in fluorescence, relative to untreated cells, indicative of elevated ROS/RNS (Figure 8B, p=0.0110). In the conditioned media samples, surprisingly, the presence of OA alone, and when combined with PEDF and H$_2$O$_2$, decreased ROS/RNS levels compared to the untreated control (Figure 8C, p=0.0261). However, when OA and H$_2$O$_2$ were added together, the ROS/RNS significantly increased compared to the control group. Similarly to RWPE-1 cells, H$_2$O$_2$ treatment did not produce the expected increase in ROS/RNS in either the intracellular or secreted environment, with the exception of the conditioned media treated with PEDF and H$_2$O$_2$.

The expression levels of mSOD, GPx-3, and GAPDH were assessed in PEDF, OA, and H$_2$O$_2$ treated LNCaP cell lysates via Western blotting and antibody hybridization. After performing densitometry, mSOD (Figure 9A) and GPx-3 (Figure 9B) expression levels were normalized to GAPDH expression within each sample. In both the presence and absence of OA, treatment with PEDF did not significantly affect the expression of mSOD or GPx-3 compared to the untreated control group or the OA-treated group.
Figure 8. PEDF treatment has no effect on LNCaP cell viability or ROS/RNS. LNCaP PCa cells were treated with PEDF (50 nM), oleic acid (OA; 1 mM), and/or H$_2$O$_2$ (10 µM). PEDF and OA treatments were 48 hours, while the H$_2$O$_2$ treatment was for the final 3 hours of treatment. Conditioned media was collected, then cells were trypsinized, an aliquot was taken for cell count, and the cell lysate was collected. (A) Cell viability was assessed on a Cellometer, and ROS/RNS levels were quantified in the cell lysate (B) and conditioned media (C) using an Oxiselect assay. * p<0.05, ** p<0.009 compared to untreated cells. Results shown are the combined data of two independent experiments.
Section C. PC-3

PEDF treatment has no effect on PC-3 cell viability

Androgen-independent PCa cells, PC-3, were treated with PEDF both with and without OA and H$_2$O$_2$. In the presence and absence of OA, the addition of PEDF had no effect on PC-3 cell viability compared to OA-alone treated and untreated, respectively (Figure 10A). PEDF also had no effect on cell viability in the presence of an additional ROS inducer, H$_2$O$_2$, compared to cells that only received the H$_2$O$_2$ treatment. Treatment with both OA and H$_2$O$_2$ did not decrease cell viability compared to untreated. However, OA treatment alone and PEDF and OA dual treatment

Figure 9. PEDF treatment does not significantly affect mSOD or GPX-3 expression in LNCaP cells in an in vitro obesity setting. LNCaP cells were treated with PEDF (50 nM), oleic acid (OA; 1 mM), and/or H$_2$O$_2$ (10 µM). PEDF and OA treatments were 48 hours, while the H$_2$O$_2$ treatment was 3 hours. The cells were trypsinized, and the cell lysate was collected. mSOD and GPx-3 expression was assessed via Western blotting of the cell lysates. Protein levels were normalized to GAPDH, and Image J software was used to assess relative mSOD (A) and GPx-3 (B) expression. No statistically significant changes were observed with PEDF treatment in the presence and absence of OA. Each experiment was performed in duplicate.
decreased viability compared to the untreated control (Figure 10A, p=0.041 and p=0.029, respectively).

**PEDF treatment does not affect oxidative stress pathways in PC-3 cells**

To measure the total ROS/RNS in PEDF, OA, and H$_2$O$_2$ treated PC-3 cells, an Oxiselect assay was performed on cell lysate and conditioned media from each sample. No statistically significant changes in ROS/RNS levels were observed in PC-3 cell lysate (Figure 10B) or conditioned media (Figure 10C). Additionally, neither cell lysate nor conditioned media treated with H$_2$O$_2$ exhibited an increase in ROS/RNS levels. The expression levels of mSOD, GPx-3, and GAPDH were assessed in the treated PC-3 cell lysates via Western blotting and antibody hybridization (Figure 11). In both the presence and absence of OA, treatment with PEDF did not significantly affect the expression of mSOD or GPx-3 compared to the untreated control group or the OA-treated group.
Figure 10. PEDF treatment has no effect on PC-3 cell viability or ROS/RNS. PCa cells, PC-3, were treated with PEDF (50 nM), oleic acid (OA; 1 mM), and/or H₂O₂ (10 µM). PEDF and OA treatments were 48 hours, while the H₂O₂ treatment was for the final 3 hours of treatment. Conditioned media was collected, then cells were trypsinized, an aliquot was taken for cell count, and the cell lysate was collected. (A) Cell viability was assessed on a Cellometer, and ROS/RNS levels were quantified in the cell lysate (B) and conditioned media (C) using an Oxiselect assay. * p<0.05, ** p<0.009 compared to untreated cells. Results shown are the combined data of two independent experiments.
Androgen-independent PCa cells, DU145, were treated with PEDF both with and without OA and H$_2$O$_2$. PEDF had no effect on DU145 cell viability compared to untreated (Figure 12A). PEDF had no effect on cell viability in the presence OA alone compared to only OA-treated cells (Figure 12A). PEDF also had no effect on cell viability in the presence of H$_2$O$_2$ compared to only H$_2$O$_2$-treated cells (Figure 12A). Dual OA and H$_2$O$_2$ treatment decreased cell viability compared to the untreated control.

Figure 11. PEDF treatment does not significantly affect mSOD or GPX-3 expression in PC-3 cells in an in vitro obesity setting. DU145 cells were treated with PEDF (50 nM), oleic acid (OA; 1 mM), and/or H$_2$O$_2$ (10 µM). PEDF and OA treatments were 48 hours, while the H$_2$O$_2$ treatment was 3 hours. The cells were trypsinized, and the cell lysate was collected. mSOD and GPx-3 expression was assessed via Western blotting of the cell lysates. Protein levels were normalized to GAPDH, and Image J software was used to assess relative mSOD (A) and GPx-3 (B) expression. No statistically significant changes were observed with PEDF treatment in the presence and absence of OA. Each experiment was performed in duplicate.

Section D. DU145

PEDF treatment has no effect on DU145 cell viability
as did OA treatment alone (Figure 12A, p=0.0245 and p=0.00137, respectively).

**PEDF treatment does not affect oxidative stress pathways in DU145 cells**

To measure the total ROS/RNS in PEDF, OA, and H\textsubscript{2}O\textsubscript{2} treated PC-3 cells, an Oxiselect assay was performed on cell lysate and conditioned media from each sample. DU145 cells treated with only H\textsubscript{2}O\textsubscript{2} or with H\textsubscript{2}O\textsubscript{2} and OA had a significant increase in intracellular ROS/RNS levels (Figure 12B, p=0.00237 and p=0.0329, respectively). OA treatment and PEDF treatment had no significant effect on ROS/RNS, although in the presence of OA, the addition of PEDF did decrease ROS/RNS compared to the OA-treated group (Figure 12B). In the conditioned media, no statistically significant changes in ROS/RNS levels were observed in DU145 samples as compared to untreated cells (Figure 12C).

The expression levels of mSOD, GPx-3, and GAPDH were assessed in PEDF, OA, and H\textsubscript{2}O\textsubscript{2} treated DU145 cell lysates by Western blotting and antibody hybridization. As measured by densitometry, PEDF alone, or with OA treatment, did not significantly affect the expression of mSOD (Figure 13A) or GPx-3 (Figure 13B) compared to the untreated control group or the OA-treated group. H\textsubscript{2}O\textsubscript{2} treatment also had no significant effect on mSOD or GPx-3 expression.
Figure 12. PEDF treatment has no effect on DU145 cell viability or ROS/RNS. PCa cells, DU145, were treated with PEDF (50 nM), oleic acid (OA; 1 mM), and/or H$_2$O$_2$ (10 µM). PEDF and OA treatments were 48 hours, while the H$_2$O$_2$ treatment was for the final 3 hours of treatment. Conditioned media was collected, then cells were trypsinized, an aliquot was taken for cell count, and the cell lysate was collected. (A) Cell viability was assessed on a Cellometer, and ROS/RNS levels were quantified in the cell lysate (B) and conditioned media (C) using an Oxiselect assay. * p<0.05, ** p<0.009 compared to untreated cells. Results shown are the combined data of two independent experiments.
Discussion

The presence of aggressive PCa is positively correlated with obesity and a high fat diet (38), suggesting that dysregulated lipid metabolism may promote PCa progression (89). One potential contributing mechanism resulting from dysregulated lipid metabolism is increased production of ROS/RNS. PEDF regulates lipid metabolism and exhibits antioxidant function in other cell types (102), and its expression is decreased in PCa (60). However, whether PEDF loss contributes to dysregulated lipid metabolism and increased oxidative stress levels has

Figure 13. PEDF treatment does not significantly affect mSOD or GPX-3 expression in DU145 cells in an in vitro obesity setting. DU145 cells were treated with PEDF (50 nM), oleic acid (OA; 1 mM), and/or H$_2$O$_2$ (10 µM). PEDF and OA treatments were 48 hours, while the H$_2$O$_2$ treatment was 3 hours. The cells were trypsinized, and the cell lysate was collected. mSOD and GPx-3 expression was assessed via Western blotting of the cell lysates. Protein levels were normalized to GAPDH, and Image J software was used to assess relative mSOD (A) and GPx-3 (B) expression. No statistically significant changes were observed with PEDF treatment in the presence and absence of OA. Each experiment was performed in duplicate.
not previously been investigated. The objective of this study was to
determine if PEDF regulates oxidative stress levels in the prostate and in
adipose tissues, operating under the hypothesis that PEDF demonstrates
antioxidant activity by reducing ROS/RNS and increasing antioxidant
enzyme levels.

*In vivo* effects of PEDF on oxidative stress

To test the effect that loss of PEDF has on oxidative stress
identifiers, ROS/RNS levels were assessed in WT and PEDF KO mouse
adipose and prostate tissues and in PEDF-treated prostate and PCa cells.
In the adipose tissue, PEDF KO mice exhibited higher levels of ROS/RNS
compared to their wildtype counterparts, with PPA and VFP tissue levels
reaching statistical significance and SQA levels approaching significance
(Figure 1A). These data support the central hypothesis of this study,
suggesting that loss of PEDF directly increases oxidative stress in these
tissues. This result is consistent with previous *in vitro* studies in granuloma
cells (102), leukemia cells (104), and retinal pericytes (109), which have
documented decreased ROS generation with exogenous PEDF treatment.
The increased ROS/RNS levels in PEDF KO adipose tissues may also be
due to the increased visceral adipose tissue surrounding the prostate (Doll
*et al.*, unpublished observations) or overall increased fat mass of PEDF
deficient mice (84), as it is known that obese adipose tissues have
increased oxidative stress levels (110). This is a likely cause of this
increase, as C57Bl/6J mice fed a high fat diet have experienced a two-fold increase in the generation of ROS in adipocytes and visceral adipose tissues (111). Additionally, C57Bl/6J mice on a high fat diet have increased fatty acid oxidation and GPx-1 expression in the liver and adipose tissues (112). In humans, higher BMI is associated with elevated lipid peroxidation and lower plasma adiponectin levels (110). Pou et al. also showed that increased SQA tissue and visceral adipose tissue is correlated with higher inflammatory and oxidative stress biomarkers (113).

In comparison to other cell types, human primary adipocytes also have increased expression of PEDF (66). This increase in PEDF expression may play a role in the regulation of oxidative stress in adipose tissues.

In PPA and VFP tissues, loss of PEDF decreased the expression of mSOD (Figure 2A); however, as tissues from only 2 mice were assessed, this did not reach statistical significance. Similar results were seen in the expression of GPx-3 in the PPA, VFP, and SQA (Figure 2B). These trends are consistent with the in vitro data presented by Amano et al., who showed that the administration of PEDF to pericytes, which are the contractile cells that wrap around endothelial cells, increased the mRNA expression of GPx-3 (109), and Zhang et al., who showed that PEDF treatment increased the expression of SOD1 protein in pericytes (114). The trend of decreased mSOD and GPx-3 expression, coupled with the increased ROS/RNS levels in the PEDF KO mice, support an antioxidant function for PEDF in these adipose tissue depots. These data support the
proposed mechanism that PEDF regulates antioxidant enzyme levels. These results are also consistent with a more recent study by Sheikpranbabu et al., who showed that PEDF inhibits the generation of ROS via increased expression of SOD and GPx in porcine retinal pericytes (115). The relative changes in both the SQA and visceral adipose tissues may be important as increased visceral adipose tissue and higher visceral adipose to SQA ratio has demonstrated stronger correlations with negative health impacts, including a higher risk for more aggressive PCa (116). Interestingly, the SQA, which exhibited the smallest change in mSOD and GPx-3 expression, also had the highest relative ROS/RNS levels (Figure 1A). Overall, these data support the hypothesis that PEDF has antioxidant function in the visceral adipose tissue depots.

While the VP, DLP, and AP tissues from PEDF KO mice showed a similar trend toward increased ROS/RNS as compared to wildtype tissues, none of these differences neared statistical significance likely due to the variance in the samples (Figure 1B). These trends are consistent with reported in vitro studies describing the decreased generation of ROS with PEDF treatment in other cell types (102, 104, 109). Interestingly, although the ROS/RNS trends in the prostate tissues are similar the results seen in the adipose tissues, the antioxidant enzyme expression levels are quite different. In the PEDF-deficient mice, only the DLP showed a notable decrease in GPx-3 expression compared to wildtype; no other tissue demonstrated a change (Figure 3B). In the VP, mSOD expression is
decreased in the PEDF KO mice, although no other tissue demonstrated a change (Figure 3A). While the trend toward an increase in ROS/RNS levels in the adipose tissues may support an antioxidant role of PED, these data suggest that the antioxidant function is not via the regulation of mSOD and GPx-3 expression levels in prostate tissue lobes. The trend toward increased ROS/RNS in the PEDF deficient VP may be due to decreased mSOD expression. In the human prostate, mSOD has increased expression in mid-grade tumors compared to noncancerous prostate (117). Future studies should expand the number of animals per group to confirm if the noted trends are statistically significant, for both the prostate and adipose tissues.

Ex vivo oxidative stress challenge

H$_2$O$_2$ is routinely used as a positive control for the induction of oxidative stress (118). In an effort to determine if absence of PEDF affected a tissue’s response to an oxidative stress inducer, a pilot study was performed comparing wildtype and PEDF KO adipose and prostate tissues responses to H$_2$O$_2$ treatment ex vivo. In the explant-cultured adipose tissues, H$_2$O$_2$ did not significantly increase ROS/RNS values. This unexpected result could be due to technical difficulties with the Oxiselect assay, such as the inability to consistently detect H$_2$O$_2$. However, as ROS/RNS levels were detected in primary untreated tissues (uncultured), this is unlikely. No previous publications describe the use of the Oxiselect
kit on H$_2$O$_2$-treated tissue, nor were any listed on the company’s website (119). Therefore, this assay may not be suitable for the detection of H$_2$O$_2$ in these tissue types. Other factors that may have affected the assay results include the concentration of H$_2$O$_2$ and the length of the treatment. While the H$_2$O$_2$ treatment was optimized on cell culture treatments, it is possible that higher concentrations are necessary for tissue treatments. It is also possible that the treatment time was not long enough to produce a measurable response. Additionally, there were no significant differences between WT and PEDF KO adipose and prostate tissues. However, the ROS/RNS levels in the PEDF KO SQA (Figure 4A) and PPA (Figure 4B) tissues were elevated compared to wildtype, although these trends did not reach statistical significance. The PPA and SQA tissue trends are consistent with the ROS/RNS data from the primary tissue assay (Figure 1A). While the trend is similar to the fresh tissue analysis, the lack of statistical significance in the ex vivo experiment is likely due to only tissues from two animals per group being tested for these two adipose tissues. Increasing the number of animals may allow for a clearer picture of the differences between these groups.

In contrast, in the VFP tissue, a small decrease in oxidative stress was observed in the PEDF KO tissue compared to the wildtype without H$_2$O$_2$ treatment (Figure 4C). This data is not consistent with the primary tissue data (Figure 1A). The discrepancy may be due to the fact that, with the ROS/RNS assay, samples were run as a single sample (i.e. not in
duplicate as with other samples) due to the small amount of tissue obtained. The PPA culture samples could not be completed in duplicate due to the extremely low weight of the harvested tissues.

Similar results were observed in the prostate tissue samples (Figure 5). The elevated ROS/RNS values for the PEDF KO tissues observed in the fresh tissue assays were not observed in the PEDF KO tissues in the ex vivo assay. However, the addition of H$_2$O$_2$ did increase ROS/RNS values in the VP, although not to a statistically significant level. This trend was evident in both the WT and PEDF KO tissues (Figure 5A). The difference seen in the VP compared to the DLP and AP may be due to the structural differences between these prostate lobes. Want et al. showed that, in TRAMP mice, over 66% of prostate tumors are found in the VP, whereas only 11.1% are in the DLP and 5.6% in the AP (120), suggesting that the VP may be more susceptible to tumor-promoting stimuli.

*In vitro* effects of PEDF on cell viability

The *in vitro* experiments in this study assessed the effect of PEDF on OA treated cells. OA stimulates triglyceride accumulation (121), which serves as an *in vitro* mimic of obesity. As with the *ex vivo* studies, H$_2$O$_2$ was added as a positive control for induction of ROS/RNS. Despite careful optimization for each cell line for optimal cell numbers for the Oxiselect assay, low levels of fluorescence were obtained and large variances within
samples were observed. Due to this high variance, few statistically significant differences were observed. However, there were discernable trends. In the absence of OA, PEDF treatment did not affect RWPE-1 cell viability (Figure 6A). However, in the presence of OA, the addition of PEDF increased cell viability compared to OA-alone treated groups, although not to a significant level. PEDF treatment alone had no effect on the viability of the PCa cell lines, LNCaP (Figure 8A), PC-3 (Figure 10A), and DU145 (Figure 12A). This result was interesting, as PEDF has shown the ability to inhibit the formation of tumors when over-expressed in PC-3 cells (57). Therefore, it is possible that PEDF exhibited very low activity in this setup. Based on the data presented here, PEDF does not have a significant effect on the viability of normal or PCa cells.

The PCa cell lines all exhibited a decrease in viability with OA treatment alone and dual OA and PEDF treatment compared to the untreated control groups, while the normal RWPE-1 cells did not. The only RWPE-1 group that experienced a significant decrease in viability was the group treated with OA and H2O2 (Figure 6A). In contrast, every treatment group that included OA, including the OA-only group, produced a decrease in viability for LNCaP (Figure 8A) and DU145 (Figure 12A). In PC-3 cells, all treatment groups with OA produced a decrease in viability except for OA and H2O2 dual-treated cells (Figure 10A). These data support that lipid accumulation actually contributes to cell death in PCa cells, while normal prostate epithelial cells are more resilient to an obesity-
challenge. These findings are consistent with a study by Hagen et al., in which they showed that a 48 hour 100 μM OA treatment significantly decreased the viability in DU145 cells and showed a trend toward decreased viability in PC-3 and LNCaP cells, although this did not reach significance (122). However, these findings are inconsistent with a study presented by Gasmi et al., in which they treated RWPE-1, LNCaP, and PC-3 cells with two isomers of oleic acid, trans-vaccenic and cis-vaccenic, at a concentration of 100 μM and found no change in viability in any of the cell lines (123). These different results could be due to different viability tests being used. In the Hagen study, a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was used, while in the Gasmi study, the WST-1 cell viability assay was used. The difference between the studies could also be due to the difference in culture times, with Gasmi et al. assessing 24-hour treatments (123), whereas Hagen et al treated cells for 48 hours (122). However, Gasmi et al. did find that five other fatty acids, jacaric acid, punicic acid, alpha-calendic acid, beta-calendic acid, and catalpic acid decreased the viability of LNCaP and PC-3, but not RWPE-1 (123). These fatty acids, which are also found in dietary sources like plant seed oils, likely induced a change in viability due to their octadecatrienoic structure; whereas, in contrast, trans-vaccenic and cis-vaccenic are both octadecaenoic (123).

Treatment with H₂O₂ at a concentration of 10 μM did not decrease cell viability for RWPE-1 (Figure 6A), LNCaP (Figure 8A), PC-3 (Figure
10A), or DU145 (Figure 12A) cells compared to untreated controls. These results are partially supported in a previous study by Freitas et al., which showed that treatment with 500 μM H₂O₂ did not affect the viability of PC-3 cells, although it did lead to a significant decrease in the viability of RWPE-1 cells (124). This difference is likely due to the higher concentration of H₂O₂ used by Freitas et al. Additionally, Bao et al. showed that treatment with H₂O₂ did not affect the viability of DU145 cells, although it did lead to a decrease in the viability of RWPE-1 cells in a dose-dependent manner (125).

*In vitro* effects of PEDF on oxidative stress

For normal prostate RWPE-1 cells, no statistically significant changes in intracellular (Figure 6B) or secreted (Figure 6C) ROS/RNS levels were detected due to the large amount of variance with the assay. However, in the presence of OA, the addition of PEDF did decrease the amount of intracellular ROS/RNS, although not to a significant level. Neither OA nor PEDF appears to influence mSOD (Figure 7A) or GPx-3 (Figure 7B), suggesting that obesity may not affect antioxidant regulation via the upregulation of these particular enzymes. These data do not support a role for PEDF in the regulation of this antioxidant pathway for normal RWPE-1 prostate cells.

For LNCaP cells, only cells treated with PEDF, OA, and H₂O₂ had a significant increase in intracellular ROS/RNS compared to the untreated
control group (Figure 8B). In the presence of OA, the addition of PEDF decreased the amount of intracellular ROS/RNS, although not to a significant level, and the addition of H$_2$O$_2$ to the OA and PEDF-treated group significantly increased intracellular ROS/RNS (Figure 8B). In LNCaP conditioned media, ROS/RNS values showed a significant decrease with PEDF and OA treatment, OA treatment alone, and OA and H$_2$O$_2$ treatment (Figure 8C). With the addition of H$_2$O$_2$ alone, secreted ROS/RNS increased compared to the untreated control (Figure 8C). OA and PEDF have no effect on mSOD (Figure 9A) or GPx-3 (Figure 9B) expression, suggesting that neither lipid accumulation nor PEDF influences antioxidant regulation via the upregulation of these particular enzymes. Combined with the ROS/RNS data, an antioxidant function for PEDF in LNCaP cells is not supported.

PC-3 cells showed no change in intracellular (Figure 10B) or secreted (Figure 10C) ROS/RNS values. OA and PEDF exhibited no effect on mSOD (Figure 11A) or GPx-3 (Figure 11B) expression. These results suggest that neither lipid-accumulation nor PEDF influence antioxidant regulation via the upregulation of these antioxidant enzymes.

DU145 cells only showed an increase in intracellular ROS/RNS with OA and H$_2$O$_2$ dual treatment and H$_2$O$_2$ treatment alone (Figure 12B). No significant changes in secreted ROS/RNS levels were observed (Figure 12C). As with RWPE-1 and LNCaP, in the presence of OA, the addition of PEDF decreased the amount of intracellular ROS/RNS,
although not to a significant level. This is, however, an interesting trend to note. The lack of effect seen with OA treatment was an unexpected result, as OA treatment was being used to simulate a mock-obesity environment in vitro. OA treatment was expected to increase oxidative stress, as obesity contributes to oxidative stress in PCa cells (126). PCa cells treated with serum from obese mice experience an increase in markers of aerobic glycolysis and ROS (127). OA was chosen for this model system due to its ability to stimulate the accumulation of triglycerides in PC-3 cells (Doll et al. unpublished observations). However, based on these findings, other fatty acids, such as punicic acid or catalpic acid may be considered for future studies. Additionally, these results are inconsistent with data presented by Bao et al., who found that treatment with 1 mM H$_2$O$_2$ increased ROS in RWPE-1 cells, but not DU145 cells (125). This discrepancy is likely due to the higher concentration of H$_2$O$_2$ used by Bao et al, which led to the elevated ROS seen in RWPE-1 cells.

While OA treatment alone increased GPx-3 expression in DU145 cells (Figure 13B), PEDF appeared to cause an increase in mSOD expression (Figure 13A). In a prospective clinical study, Li et al. showed that the risk of aggressive PCa is increased in the presence of a somatic mSOD polymorphism that decreases enzymatic function, supporting a role for mSOD in PCa tumor progression (128). Similarly, Yu et al. reported that GPx-3 is inactivated in PCa, and overexpression of GPx-3 in PCa cells led to their reduced invasive potential (129).
The overall ROS/RNS values and mSOD/GPx-3 expression data from the prostate and PCa cells presented here do not seem to support an antioxidant effect of PEDF in the prostate. There were no changes in the antioxidant enzyme expression levels, except for in the DU145 cell line, in which PEDF increased mSOD expression. PEDF treatment of prostate and PCa cells induced no changes in ROS/RNS levels, further supporting this lack of anti-oxidant function in the prostate. Additionally, the mock obesity treatment of OA did not induce a change in mSOD and GPX-3 expression, with the exception of in DU145 (Figure 13), where OA increased GPx-3 expression. However, this overall lack of effect seen with OA treatment was surprising, as OA treatment was expected to increase oxidative stress due to the previously reported contribution to oxidative stress by obesity in PCa cells (126). While our studies do not necessarily support a link between PEDF and antioxidant enzyme levels in the prostate, some studies have shown a link between a high fat diet and decreased antioxidant enzyme expression. In TRAMP mice, a transgenic prostate cancer model, a high fat diet led to a decrease in GPx-3 expression in the DLP and AP as compared to animals on a control diet (130).

It has been noted that there are several evident differences in the results between the three PCa cell lines used in this study. Despite the fact that LNCaP, PC-3, and DU145 are all cells derived from human PCa tumors, there are distinct genetic differences between these cell lines,
most notably with the well-characterized tumor suppressor genes, PTEN, P53, and Rb. LNCaP produces non-functional PTEN (131) protein, but normal p53 (132) and Rb protein (133). In contrast, PC-3 produces no PTEN (131) or P53 (132) protein, but normal Rb protein (133), and DU145 produces functional PTEN protein (131), but non-functional P53 (132) and Rb protein (133). The presence, absence, or loss of function of these prominent anti-tumor proteins may affect the cellular responses to the given treatments and lead to the observed varying results between the cell lines.

Overall, the high variability in the data of the cell treatments is likely due, in part, to the Oxiselect assay. This assay was first introduced in 2010 (119) and was also a novel assay to our lab. While the manufacturer’s instructions appear straightforward, several technical difficulties were experienced in optimizing and completing the assays for these studies. Most notably, despite several optimization trials to determine the most appropriate concentration of cells needed to detect ROS/RNS, concentration levels were often on the low end of the spectrum, below the quantification values of the standard curve, but well above background levels. This is why the values for the in vitro studies are reported as fluorescence values, relative to the untreated groups. However, as fluorescence is a direct by-product of ROS/RNS in this assay, these values are still appropriate representations of oxidative stress levels within the cell lysate and conditioned media groups. In
contrast, homogenates from fresh tissue produced calculable ROS/RNS concentration values. No published studies reporting similar problems with low ROS/RNS readings with this assay were found with a literature search. Additionally, no reports on the use of this assay on PCa cells were identified.

Another unexpected result observed in the Oxiselect assay data is that the addition of H$_2$O$_2$ did not significantly increase the ROS/RNS values of RWPE-1, LNCaP, and PC-3 cells, despite the fact that H$_2$O$_2$ is an inducer of oxidative stress. It is possible that the concentration of H$_2$O$_2$ was not high enough to cause a significant change in the cells. Despite the fact that in several optimization trials run prior to these experiments, ROS/RNS levels with the concentration of H$_2$O$_2$ used were detectable. The difference between the optimization trials and the experimental trials were the number of cells per plate when the 3-hour H$_2$O$_2$ treatment was added. For the optimization trials, the treatment was added when plates were near confluent, although no cell counts were conducted prior to treatment. For the experimental trials, cells were plated at 25,000 cells/cm$^2$ and left overnight before adding the treatments the next morning. Therefore, the difference in cell density on the plate may be responsible for these varying results. Nonetheless, future studies of this nature should assess the effect of cell number on ROS/RNS levels as well as test higher concentrations of H$_2$O$_2$. 
Conclusions and Future directions

The purpose of this study was to determine if PEDF has an antioxidant function in adipose tissue and prostate, including in PCa. The data presented in this thesis partially support the central hypothesis, in that loss of PEDF in vivo led to a significant increase in total ROS/RNS levels in murine adipose tissue. These adipose tissues also had trends of lowered antioxidant enzymes (mSOD and GPx-3) further supporting an antioxidative role for PEDF. PEDF loss in normal prostate tissues led to an increase in total ROS/RNS in the prostate, although not to a statistically significant level. However, similar results were not observed in the normal prostate or in PCa cells in vitro. In these studies, PEDF did not appear to influence total ROS/RNS or antioxidant expression levels, except for the increase of mSOD in DU145 treated cells. Further studies are needed to validate PEDF’s antioxidant function in the adipose tissue and to determine if the trends seen in the prostate and PCa cells are significant. In particular, the in vivo studies should be repeated with larger group numbers. Increasing the number of mice may help to determine if the trends seen in the expression levels of mSOD and GPx-3 were statistically significant. In addition, as these data do not support a strong role for GPx3 or mSOD in the prostate, other antioxidant enzymes, such as catalases or peroxiredoxins, should be investigated in future studies to attempt to
better understand the underlying molecular mechanisms responsible for the increased oxidative stress in PEDF deficient mice.

Several adjustments should also be made in the *in vitro* experiments for future studies; a higher concentration of $\text{H}_2\text{O}_2$ should be used, and other fatty acids should be used to simulate an oxidative stress-inducing obese environment. A well-established antioxidant, such as vitamin C or vitamin E, could also be introduced into the treatment system to determine if there is the expected decrease in oxidative stress. These future studies would help to expand upon this work and further establish a full antioxidant role for PEDF. Overall, the work presented in this study does support that PEDF has an antioxidant function in adipose tissue, although its role in the prostate remains unclear. It also supports the justification of the outlined future experiments towards better establishing PEDF’s function in obesity-mediated PCa progression.
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