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Metformin-Induced PEDF Expression Regulates Cell Proliferation and Lipid Metabolism in Prostate Cancer Cells

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**METFORMIN-INDUCED PEDF EXPRESSION
REGULATES CELL PROLIFERATION AND LIPID
METABOLISM IN PROSTATE CANCER CELLS**

by

Miguel Tolentino

A Thesis Submitted in
Partial Fulfillment of the
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August 2013

ABSTRACT
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PROLIFERATION AND LIPID METABOLISM IN PROSTATE CANCER
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Miguel Tolentino

The University of Wisconsin-Milwaukee, 2013
Under the Supervision of Jennifer Doll, PhD

Prostate cancer (PCa) is one of the most common cancers in American males. A high fat diet and obesity accelerate PCa progression and increase the risk of death from disease. Epidemiological studies have indicated that PCa patients with type 2 diabetes have higher mortality rates than PCa patients without diabetes. Type 2 diabetics who are on metformin, a drug to control blood sugar levels, show a delay in PCa progression in comparison with PCa patients with type 2 diabetes who are not on metformin. It has been proposed that metformin inhibits proliferation via activation of AMP-activated protein kinase (AMPK) or by blocking proliferation at the G₀/G₁ phase in the cell cycle with a reduction in cyclin D1. However, the mechanism is still unclear, as these *in vitro* studies used 5 mM metformin which is physiologically not attainable. AMPK is known to stimulate lipolysis, the breakdown of triglycerides into fatty acids functions. As increased lipid anabolism is known to promote PCa, the lipolysis produced by AMPK, during metformin treatment, may also contribute to the anti-tumor activity of metformin.

Pigment epithelium-derived factor (PEDF) is a glycoprotein with multiple functions. Its expression is decreased in many cancer types, including PCa. Exogenous PEDF treatment reduces prostate tumor growth, metastasis, and angiogenesis. Interestingly, it has been reported that type 2 diabetes patients on metformin present with higher serum levels of PEDF in comparison with healthy patients not on metformin.

In this work, I tested the hypothesis that one mechanism of metformin's activity may be via stimulation of PEDF. Metformin increased intracellular PEDF in LNCaP cells, and extracellular PEDF in RWPE-1 and LNCaP cells. Metformin also reduced cell proliferation and increased lipolysis in PCa cells at physiological obtainable doses (0.02 and 0.5 mM). I also observed that at these lower doses, metformin increased the expression of cyclin D1 and AMPK, but at 5 mM doses reduced cyclin D1. Supporting PEDF's role in lipid metabolism, in PEDF KO prostate tissues there was less lipolytic activity and altered triglyceride levels in comparison with wild type tissue. Overall, these data support the hypothesis that metformin may act through PEDF.

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INTRODUCTION

Prostate cancer

Prostate cancer is a disease that affects older men. Epidemiological studies have reported that 65% of prostate cancer patients are diagnosed over the age of 65 [1]. The concern about prostate cancer has increased because it has become the second leading cause of death by cancer after lung cancer in American men [2]. The prostate is a male reproductive gland, approximately 4 cm in width, and is localized under the urinary bladder, in front of the rectum. Its main function is the production of seminal fluid. The prostate is constituted by three glandular regions called, the peripheral zone, central zone, and transition zone. There is a fourth region which is not glandular called the anterior fibromuscular stroma [3, 4]. Approximately 95% of prostate cancers are adenocarcinomas, which are cancers that originate from the prostatic epithelial cells. Of these 70% are localized in the peripheral zone, 15-20% in the central zone, and 10-15% in the transitional zone [1, 4]. Cancers metastasize via the lymphphatic system and through the bloodstream to reach distant sites, and in metastatic prostate cancer, common sites of metastasis are the bone and brain [1, 4].

The initial stages of prostate cancer are asymptomatic. Most of the time, symptoms are present only when the disease is in a very advanced stage. Some symptoms include dysuria (pain during urination), slow stream of urine flow, incontinence, and urine retention. Symptoms during metastasis include,

pancytopenia (a reduction in the production of erythrocytes, white blood cells, and platelets), anemia, weight loss, and leg pain [5].

The diagnosis of prostate cancer is often difficult. Digital rectal examination (DRE) and the prostate specific antigen (PSA) blood test are the two main tests used to identify prostate cancer. DRE possesses a low sensitivity with approximately 54% accuracy. However, this test can detect prostate cancer in men with normal values of PSA, and it is cheap, usually acceptable among patients, and can also detect benign prostatic hyperplasia (BPH). PSA is the most common test used to detect, classify, and monitor prostate cancer. The PSA blood test is an inexpensive test, with acceptance among patients, and high sensitivity, but is not very specific. High levels of PSA can be detected also in pathological conditions such as BPH and prostatitis [1]. If either of these tests indicates the presence of cancer, then transrectal ultrasound imaging is used to guide the acquisition of biopsy tissues from the prostate. Thus, to obtain an accurate diagnosis, it is necessary to evaluate symptoms, medical history of the patient, and also use other tests such as biopsy and histological examination. In cases where metastatic cancer is suspected, X-rays, magnetic resonance imaging, computerized tomography scans, and bone scans would also be used [1].

Once a tumor is diagnosed, it is characterized by stage and grade. For staging, the tumor, node, metastasis (TNM) system is used. The TNM classification system depicts the characteristics of the tumor (T), if the cancer has spread to the regional lymph nodes (N), or if the cancer has metastasized (M) [1]. The tumor grading of prostate cancer is a tool which relies on histologic assessment of the prostate tissue. The degree of malignancy of a tumor can be determined according to its physical characteristics under

the microscope. The Gleason score is the most common grading system to classify prostate cancer. The Gleason system is based on the examination of prostate tissue obtained by biopsy or following surgical removal of the prostate. In this system, histological patterns are classified by the appearance of the glands and cells and scored on a 1-5 scale. As prostate occurs in the glands and is heterogeneous, the Gleason score is then based on the score obtained after the sum of the 2 most predominant patterns. As general rule, a score under 6 is not considered cancer, while a score of 6 or above is cancer, and a score at or above 7 indicates an aggressive cancer [6, 7].

Prostate cancer progression and angiogenesis

Prostate cancer begins as a consequence of irreparable damage in the DNA, leading to both abnormal growth and cell functions. The carcinogenesis process involves three phases: initiation, where the DNA damage is initiated; promotion, where DNA damage produces cellular changes in prostate cells; and progression, where both an increase in the number of cells and an abnormal performance occur [8].

For cancer progression and metastasis, it is necessary that the tumor induces its own blood supply. In order to do this, the tumor uses a process called angiogenesis. Angiogenesis is the formation of new vessels from existing vessels [9, 10]. This process is regulated by multiple factors. In normal tissue, for example, there are proteins that inhibit the angiogenic process. Conversely, angiogenesis can be activated by the presence of pro-angiogenic factors such as vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), fibroblast growth factor (FGF-2), and interleukin-8 [9, 10]. The angiogenesis process in tumors can be blocked by the inhibition of these pro-

angiogenic factors or introducing anti-angiogenic molecules, and thus could be a safe and less toxic therapeutic strategy against cancer progression [9, 10]. It has been reported that pigment epithelium-derived factor (PEDF), a multifunctional angiogenesis inhibitor, is down-regulated in several cancers [11].

Pigment epithelium derived factor (PEDF)

PEDF is a 50 kDa glycoprotein that was identified in 1987 from cell conditioned media from retinal pigment epithelial cells [12]. PEDF belongs to the serine protease inhibitors (Serpins) family of proteins. The Serpins family includes two groups. Both Serpin groups share similarities in structure; however, the first group possesses a region called the reactive center loop which contains a region that interacts with the specific protease. The second group does not have this motif and thus does not function as a protease [13]. PEDF is a member of the second group, and some of this group's functions include hormone transport, molecular chaperone, and tumor suppression [13].

PEDF possesses four main functions: angiogenesis inhibition, promotion of apoptotic activity, neuroprotective activity, and induction of cell differentiation. These functions are cell type specific [14]. Two peptide regions (epitopes) that mediate many of PEDF functions have been identified. The 44-residue fragment (residues 78-121) is responsible for the anti-angiogenic and tumor cell apoptosis activities, while the 34-residue fragment (residues 44-77) is responsible for the neuroprotective effect and cell differentiation activity [12, 14, 15].

In the eye, PEDF promotes homeostasis and, in pathological conditions, it promotes apoptosis in endothelial cells to avoid the aberrant formation of blood vessels

[16]. Wang, *et al*, reported that exogenous PEDF treatment reduced proliferation of human aortic smooth muscle cells (HASMCs) [17]. In fact, PEDF has presented a higher anti-angiogenic effect than other angiogenesis inhibitors such as angiostatin, thrombospondin-1, and endostatin [14]. In *In vitro* studies, it was found that PEDF blocks cell cycle progression at the G₀/G₁ phase and reduces cyclin D1, cyclin E, CDK2, CDk4, and p21 in endothelial cells [17]. It was shown in *in vivo* studies using athymic mice with neuroblastomas that intratumoral injections of recombinant PEDF generated cell differentiation and lead to the production of fewer malignant cells in histological samples [18].

PEDF in prostate cancer

In the prostate, the gene that encodes PEDF, SERPINF1 plays a fundamental role as a tumor suppressor gene. It has been found that PEDF knockout (KO) mice present with epithelial cell hyperplasia and higher stromal microvessel density in the prostate as compared to the prostate of wild type mice [19].

Doll, *et al*. has previously found that PEDF expression is down-regulated in prostate cancer patient tissues, and is also down-regulated in three prostate cancer cell lines (DU145, LNCaP, and PC-3) in comparison with a normal prostate epithelial cell line [19]. Furthermore, the treatment of tumor xenografts with recombinant PEDF reduced stromal vasculature and induced apoptosis [19]. *In vitro*, PEDF induced apoptosis of prostate cancer cells, and also it has been found that PEDF is down-regulated by androgens [19].

Guan, *et al*, reported the use of an adenoviral vector to induce the overexpression of PEDF in PC-3 cells. They found that overexpression of PEDF produced a reduction in cell proliferation [20]. Moreover, microarray analysis showed that PEDF regulated genes involved in signal transduction, proliferation, angiogenesis, and apoptosis [20]. In another study, DU145 and PC-3 cells were treated with exogenous PEDF, and genes that were up-regulated in both cell lines included a brain angiogenesis inhibitor, a binding adaptor protein (adaptor-related protein complex 2, mu 1 subunit), and an apoptosis regulator (growth arrest and DNA-damage-inducible, alpha). Genes that were down-regulated in both cell lines included AMP-activated protein kinase (AMPK), ubiquitin specific protease 6, and fibroblast growth factor 3 [21]. These data emphasize the multifunctional nature of PEDF and its potential as a therapeutic target.

PEDF and lipid metabolism

It has been suggested that PEDF also plays a role in lipid metabolism. In a published study, it was found that PEDF was synthesized *in vitro* by human primary adipocytes obtained from lean or moderately overweight patients at a concentration of 130 ng/ml over 24 h from one million cells [22]. This concentration is relatively higher compared to other molecules synthesized by the adipocytes such as adiponectin, interleukin-6, or IL-8 [22]. In mouse models, an increase in serum levels of PEDF was detected during obesity, while a reduction was observed during weight loss [23]. Moreover, it has been reported that type 1 diabetes patients with microvascular complications and, also, type 2 diabetes patients present with higher serum levels of PEDF in comparison with non-diabetic controls [24, 25]. In the adipose tissue, the anti-

angiogenic PEDF maintains homeostasis and prevents over vascularization during adipose tissue growth by the inhibition of angiogenic factors such as TNF- α , PDGF, VEGF, and FGF-2 [23].

In hepatocytes, PEDF binds to adipose triglyceride lipase (ATGL) to stimulate the breakdown of triglycerides [26, 27], and the lack of PEDF and/or ATGL produces lipid accumulation [26]. These results indicate that PEDF participates in lipid metabolism; however, the molecular pathway and precise role is not clear. In addition, the lipid regulatory functions of PEDF have not been studied in prostate cancer cells.

Obesity, high fat diet, and prostate cancer

Epidemiological studies have reported that obesity and a high fat diet increase the incidence and risk of death from disease in many cancer types, including esophageal, colon, rectal, liver, gallbladder, pancreatic, renal, breast, and ovarian cancers [28]. In prostate cancer, while obesity and a high fat diet do not increase the risk of disease, they do increase the risk of death from disease [1]. Epidemiological studies have found that obese prostate cancer patients have a higher mortality rate than patients of normal weight [29]. The effect of a high fat diet, also called a Western diet, on prostate cancer can be observed in epidemiological studies of Japanese and Chinese populations. The diet of these Asian populations is characterized by high levels of fiber and low levels of fats, and both populations present with low incidence of prostate cancer [30]. However, after migration to the United States, both populations experience an increase in prostate cancer incidence [30-32]. Studies in mouse models have shown that the consumption of a diet with high levels of fat and cholesterol accelerates cancer progression and angiogenesis

[2]. For example, a high fat diet increases the cell growth of LNCaP cells in a xenograft model [33]. On the other hand, a low fat diet and a high-fiber diet decrease the cellular growth and increases apoptosis in LNCaP cells [34]. These data demonstrate that environmental factors, such as a change in diet or other lifestyle changes (consumption of drugs, alcohol, tobacco, etc.), affect prostate cancer.

Diabetes, metformin, and cancer

Obesity significantly increases the risk of a patient developing diabetes. Epidemiological studies have indicated that diabetics have a higher risk of acquiring cancer and also have a higher mortality rate than non-diabetic cancer patients [29, 35-37]. In prostate cancer, diabetes is associated with a worse prognosis and a higher mortality rate than non-diabetic prostate cancer patients [29, 35]. Type 2 diabetes patients often present with insulin resistance which leads to hyperinsulinemia [38]. As stated previously, obesity increases the risk of developing some kinds of cancers, and insulin is a hormone related with growth processes promoting cell proliferation. Thus, it has been suggested that obesity combined with high levels of insulin produce a carcinogenic effect [39].

Interestingly, cancer patients with type 2 diabetes on a drug called metformin have shown a slower cancer progression [35, 39]. Metformin is a drug commonly used by type 2 diabetes patients in order to control their blood sugar levels. It is absorbed into the body within 1-3 hours after administration, and 90% is eliminated by the renal system [40]. Metformin produces several different effects. It reduces circulating glucose levels by decreasing the production of glucose by the liver. It also reduces the resistance to

insulin by increasing the uptake of glucose by muscles and adipose tissue, resulting in normalization of insulin levels [39]. The effect of metformin on reducing glucose levels is observed only in patients with diabetes and/or insulin resistance, but metformin does not have an effect on healthy people [40].

It has been reported that metformin decreases the incidence of prostate, pancreatic and colon cancers, and decreases the progression of prostate, breast, pancreatic, colon, lung, and ovarian cancers [35, 39, 41]. Epidemiological studies compared the mortality rate in diabetic prostate cancer patients on insulin, sulfonylureas (sulfonylureas increase insulin secretion) or metformin, and in non-diabetic prostate cancer patients. The results showed that diabetic patients on insulin or sulfonylureas therapy had a higher mortality rate in comparison to non-diabetic patients, and patients on metformin showed a reduction in mortality [35, 36]. Thus, it has been suggested that metformin may possess anticancer effect in prostate cancer; however, its molecular pathways have not been studied completely.

As explained above, there is a decrease in PEDF expression in prostate tissue [19] and in circulating levels in prostate cancer patients [42]. In contrast, obese type 2 diabetes patients have high serum levels of PEDF [25], which appears to correlate most closely with insulin resistance [22]. However, the tissue source of this change in circulating PEDF is currently unknown, and studies in obese prostate cancer patients have not been reported. While some studies indicate that PEDF participates in the resistance to insulin [22], other studies indicate that the increase in the PEDF levels is a compensatory response to high insulin levels and insulin resistance [23]. Thus, further studies are

necessary to elucidate the PEDF's role in diabetes and to identify the tissue that produces the high values of PEDF in the serum of diabetics.

Metformin and lipid metabolism

Most studies on the mechanism by which metformin treats diabetes have been focused on the liver. In hepatocytes, metformin activates AMPK, which regulates lipid and glucose metabolism [43]. The activation of AMPK in the liver produces a reduction in the activity of acetyl-CoA carboxylase (ACC), which is used to convert acetyl-CoA into triglycerides, and also increases fatty acid oxidation (breakdown of fatty acids into acetyl-CoA in the mitochondria to produce energy), which results in a reduction in lipids stores [43]. It is known that many cancer cell types have high levels of *de novo* fatty acid synthesis which participates in cell proliferation and cell survival, including that of prostate cancer [44]. Therefore, a reduction in the lipid stores can affect the cancer cells that are characterized by a high lipogenic metabolism, reducing their activity and expression of oncoproteins [45].

AMPK activation also inhibits the mammalian target of rapamycin (mTOR), a protein that participates in the control of cell energy expenditures by the regulation of protein synthesis, cell growth, and autophagy. Activation of the mTOR pathway promotes tumor growth in several cancer types; its activation correlates with cancer progression, adverse prognosis, and chemotherapy resistance [40]. Thus, the inhibition of mTOR by metformin via AMPK can be one possible pathway for the antiproliferative effect of metformin, according to studies related with renal and breast cancer [39, 40]. In addition, *in vitro* experiments using human THP-1 macrophages have shown that

metformin reduced intracellular lipid accumulation and reduced the expression of fatty acid-binding protein 4 (FABP4), a protein which functions in fatty acid uptake. Interestingly, this protein is also expressed by PC-3 prostate cells [46, 47].

Metformin, prostate cancer, and PEDF

The effect of metformin on prostate cancer cells has been studied, showing that metformin reduced cell proliferation in LNCaP, DU145, and PC-3 cells [48-50]. The main drawback of these studies is that the authors used high doses of metformin on a small number of cells. Moreover, they used colorimetric assays [48, 50] to evaluate cell proliferation or viability instead of a direct cell counting method. In one of these published studies, the authors showed that metformin at 1 and 5 mM doses reduced cell proliferation in LNCaP, DU145, and PC-3 prostate cancer cells [50]. They found that the 5 mM metformin dose inhibited cell proliferation in LNCaP and DU145 prostate cancer cell lines by blocking the G₀/G₁ phase during the cell cycle, which correlated with a diminution of the cyclin D1 levels, which is required in the G₁ phase [50]. This activity was not seen in PC-3 cells.

Furthermore, the authors found that metformin activated AMPK in all three prostate cancer cell lines. However, they found, using siRNA against AMPK, that the anti-proliferative effect of metformin was still present in DU145 and LNCaP cells [50]. This suggests that AMPK is not the pathway for the antiproliferative effect of metformin at these doses. A caveat of the above studies is that it is not possible to achieve 1 and 5 mM levels of metformin physiologically in human patients. Current prescribing information indicates that metformin is recommended in doses in the range of 500 mg to

2000 mg once daily, reaching plasma levels of approximately 0.6-1.8 $\mu\text{g/mL}$ (NDA 20-357/S-031, NDA 21-202/S-016). Thus, the highest physiologic concentration expected for metformin is around 10.8 μM . Therefore, the use of physiologically obtainable doses of metformin is a critical point to study the effect of metformin in humans.

In other cell types, PEDF has been shown to decrease cyclin D1 levels in other cell types [17]. Another published study reported an increase in serum levels of PEDF in newly diagnosed type 2 diabetes patients after metformin treatment. In this study, 36 type 2 diabetes patients received a dose of 850 mg of metformin twice a day. After six months, serum PEDF levels of patients on metformin were significantly higher than those in patients who were not on metformin [51]. This study supports a relationship between metformin and PEDF. Thus, it is possible that metformin exerts its anti-proliferative effect through induction of PEDF expression with a consequent reduction of cyclin D1 and/or lipid metabolism. The effects of PEDF on lipid metabolism have not been studied in prostate cancer cells. Determining if metformin activity works in part through PEDF could potentially open new possibilities to anticancer treatments targeting this pathway. Thus, the above studies led to my hypothesis that metformin exerts its anti-proliferative and lipid-regulatory functions in part via inducing PEDF expression in prostate cancer cells. In order to support the hypothesis, the following specific aims were evaluated:

Specific aim 1. Elucidate if metformin mediates its anti-proliferative activity through PEDF in the presence or absence of lipid overload by:

- a) Quantifying PEDF level in metformin with or without OA treated prostate cells;

- b) Assessing levels of cyclin D1 and activated AMPK in metformin with/without OA treated cells; and
- c) Establishing if anti-PEDF siRNA treatment blocks metformin's anti-proliferative effect.

Specific aim 2. Examine if metformin regulates lipid metabolism in prostate cancer cells by:

- a) Assessing lipid levels qualitatively [oil red O (ORO) staining technique] and quantitatively (direct triglyceride quantification) in metformin treated cells with and without OA treatment;
- b) Quantifying the lipolytic activity in samples from 2a; and,
- c) Examining the levels of lipolytic activity in explant cultures of prostate tissues from wild type versus PEDF knockout mice and quantifying triglyceride levels in these tissues.

MATERIALS AND METHODS

Cell lines and culture conditions

DU145, PC-3, LNCaP, and RWPE-1 cell lines were purchased from the American Type Culture collection (ATCC, Manassas, VA). These cell lines are commonly used to study prostate cancer. DU145 is a cell line that was isolated from a human prostate adenocarcinoma metastasis to the brain [52]. PC-3 is a cell line that was obtained originally from a human prostatic adenocarcinoma metastasis to bone [53]. LNCaP is from a metastatic lesion of human prostatic carcinoma in lymph node [54]. RWPE-1 is a normal adult human prostatic epithelial cell line, from a male donor, that has been immortalized using human papilloma virus 18 [55]. The prostate cancer cell lines DU145 and PC-3 were grown in Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich, Saint Louis, MO, Cat.# D5796) with 10% fetal bovine serum (FBS, Sigma-Aldrich, Saint Louis, MO, Cat # F2442) and 1% penicillin/streptomycin (P/S, Cellgro, Manassas VA, Cat.# 30-002-CI). The LNCaP cell line was grown in Roswell Park Memorial Institute Medium (RPMI) medium with 10% FBS and 1% P/S. RWPE-1 cells were grown in keratinocyte complete media with 1% P/S (Cellgro, Manassas VA, Cat.# 30-002-CI). The cells were all grown at 37°C and 5% CO₂.

In Vitro obesity experiment using oleic acid

For *in vitro* experiments, cells were plated at 20,000 cells/cm² on tissue culture dishes (10 cm dishes, 6 well plates or 2 well chamber slides) at 37°C, 5% CO₂ overnight. After incubation the growth medium was aspirated and the cells were washed with sterile PBS. Then, treatment medias (Table 1), with metformin at different doses with or without

cell number, and cell viability for each count were recorded. To detect changes in cell proliferation each treatment was compared with untreated.

Cell lysate collection

Cell lysates were used to determine PEDF, cyclin-D1, and AMPK levels after treatment with metformin with or without OA. Once the cell proliferation assays were performed, the cells collected were pelleted by centrifugation at 800-1000 x g for 8 minutes, and the supernatant was aspirated. Cell lysis buffer (1X, 350 µl, Cell Signaling, Danvers MA, Cat.# 9803S) was added and mixed by pipetting. The cell mixture was then incubated on ice for 5 minutes, and then vortexed for 15-30 seconds. The cell lysate solution was then transferred to a microfuge tube and centrifuged at 14000 x g at 4°C for 10 minutes. The supernatant was collected into siliconized tubes. Samples were stored at 4°C for immediate use or at -80°C for long term storage.

Collection and concentration of conditioned media

The conditioned media (CM) collected above was centrifuged (800 x g for 8 min) to pellet any cellular debris, and the CM was transferred to a new tube. If needed for a lipolysis assay, an aliquot was taken out. Then, 50 µL of 1X protease inhibitor cocktail (Sigma-Aldrich, Saint Louis MO, Cat.# P8340) and 50 µL of 100 nM phenylmethanesulfonyl fluoride solution (PMSF, Sigma-Aldrich, Saint Louis MO, Cat.# 93482) were added for a final 1:100 dilution of protease inhibitor cocktail and 10 µM PMSF. The conditioned media were concentrated using a Millipore ultra-15 centrifugal filter device with a 3 kDa cutoff and 15 ml volume (Amicon Ultra, Billerica, MA, Cat #

UFC900324). Briefly, the membrane of the device was pre-wet using 5 ml of sterile PBS and a spin of 4000 g at 4°C for 10 minutes. The remaining PBS was removed, and the conditioned medium was added to the membrane approximately 10 mL at a time and spun at 4000 g approximately 30 minutes interval. The concentrating continued at 5-60 minute intervals until all media from samples were concentrated. The filtrate was discarded after each spin and more conditioned medium was added. After the addition of the entire conditioned medium, the concentrated conditioned medium was washed with 1-2 volume of PBS or until the phenol red color disappeared. The conditioned media were concentrated approximately 10 fold (a 500 μ L of volume from 5 ml of conditioned medium). The concentrated conditioned media were transferred to a sterile siliconized microfuge tube and stored at -80°C until needed.

Qualitative assessment of lipid accumulation

In order to assess lipid accumulation qualitatively, cells on chamber slides were stained following the protocol of nova UltraTM Oil Red O (ORO) stain kit (IHC World, Woodstock MD, Cat.# IW-3008). Briefly, the cells were plated onto chamber slides at 20,000 cells/cm² as described above. For the RWPE-1 and LNCaP cells to enhance cell attachment to the slides, the slides were pre-coated with a 0.1% solution of Difco gelatin overnight at 4°C (Becton Dickinson, Sparks, MD, Cat.# 214340). After incubation, the gelation solution was aspirated and the slides were washed once with PBS. Then, the cells were plated and treated as described above. After 48 h of treatment, the media were aspirated and the cells were rinsed once with PBS. The chamber cassette was removed with a razor blade and the cells were fixed using 10% formalin for at least 10 minutes.

The slides were rinsed 3 times with distilled water and then placed in pre-stain solution for 3-5 minutes. Next, the slides were stained in pre-warmed ORO solution at 60°C for 10 minutes. Next, the slides were placed in differentiation solution for 3-5 minutes, and rinsed 3 times with tap water. The slides were placed in Myer's hematoxylin solution for 1 minute to stain cell nuclei. Slides were rinsed with distilled water once, and coverslips placed over the cells using aqueous mounting medium (GBI Labs, Mukilteo, WA, Cat.# E01-18). After staining, the lipids stained red and the nuclei stained pale blue. Changes in lipid accumulation were evaluated under a microscope at 40X and photographed as a record of the data.

Quantitative assessment of lipid levels

After metformin with/without OA treatments, one set of cell lysates for each cell line was collected for quantification of triglyceride levels. The protocol for triglyceride isolation used is a modification of the Folch technique [26]. Briefly, after the metformin with/without OA treatments, the conditioned media were removed and the cells were washed 3 times with 3-5 ml of cold PBS. The cells were trypsinized and transferred to 15 ml conical tubes using 2 volumes of growth media. The cells were centrifuged at 800 x g for 10 min, the media were aspirated, and the cells were resuspended in 500 μ L of PBS. The cells were then transferred into 1.5 ml microfuge tubes, and each sample was sonicated two times for 25 seconds at 40% amplitude (Sonics, Sonis Vibra Cell, model No. VCX130PB, Newtown, CT,). The samples were then transferred to glass tubes and 1.90 ml of 2:1 CHCl_3 :MeOH was added. The samples were incubated 15 minutes at room temperature (RT) and vortexed every 5 min. Next, 0.6 ml of CHCl_3 and 0.5 ml of NaCl

were added and then the sample was vortexed. The samples were spun at $1910 \times g$ (4000 rpm) for 30 min at 4°C . The upper aqueous layer was aspirated and the protein disk was removed. The samples were maintained on ice, and the organic phase was dried under nitrogen gas. Each sample was resuspended in 60 μL of CHCl_3 , and 20 μL were transferred in a new glass tube and dried again under nitrogen gas.

Fifteen μL of autoclaved mH_2O were added to the 20 μL dried sample. To each sample, 1 mL of triglyceride reagent (StanBio, Boerne TX, Cat.# 2201-030) was then added. The mixture was vortexed and incubated 10 minutes at RT. A serial dilution of triglyceride standard (StanBio, Boerne TX, Cat.# 2103-002) was prepared. Two hundred μL of each sample and standards were placed in triplicate wells in a 96 well plate. The plate was read on a plate reader (Bio-Tek, model KC4, Winooski, VT) at 500 nm. Triglyceride levels were determined by comparison to the standard curve. Data were normalized to total cell number (for cells) or tissue weights (for prostate tissues; described below).

Collection of mouse prostate tissues secretions.

Mouse prostate tissues were obtained following the general procedures established by Dr. Doll (described below) and approved by the Institutional Animal Care and Use Committee of the University of Wisconsin-Milwaukee. The ventral, anterior, and dorsolateral lobes prostates from the wild type ($n=5$) and PEDF-KO ($n=5$) mice were collected in order to determine lipolytic activity and the triglyceride content of the mouse prostate for each strain. Briefly, the mouse was anesthetized with isoflurane inside a desiccator jar. After anesthesia, the mouse was euthanized by cervical dislocation and

placed in a dorsal position. Next, using sterile scissors and forcep, a midline incision was made through the abdominal wall and peritoneum. The intestines were moved to side to reveal the reproductive system. Finally, the anterior, ventral and dorsolateral lobes of the prostate were excised under a dissecting microscope. Tissues were either immediately frozen in liquid nitrogen or placed on ice for explant culture.

For explant culture, the weight of each prostate lobe was recorded. Then each tissue was transferred to a 10 cm tissue culture dish and washed 3 times with 1 ml of PBS. For dissection of tissues, sterile forceps, scalpel blades, or scissors were used. The tissue was dissected in 1-2 mm pieces and placed in a well of a 6 well plate with 1 ml basal media (DMEM). The plate was incubated at 37°C with 5% CO₂ for 48 hours. After incubation, the conditioned media were collected. The conditioned media were used in the lipolysis assay, as described below.

For the triglyceride quantification, each tissue was weighed and placed in a glass tube, washed with PBS, and homogenized with 300 µL of 2:1 CHCl₃:MEOH solution using a Kontes motor and disposable pestles (Fisher Scientific, Waltham, MA, Pestles KT-749521-1590, Kontes motor KT749540-0000). To each sample, 100 µL of CHCl₃ and 75 µL of NaCl were added and vortexed. The samples were then centrifuged at 1910 x g (4000 rpm) for 30 min at 4°C. The upper aqueous layer and protein disc were aspirated. The samples were maintained on ice and the organic phase was dried using nitrogen gas. Each sample was resuspended in 60 µL of CHCl₃, and 20 µL were transferred to a new glass tube and dried again under nitrogen gas. The dried samples were used for the triglyceride quantification assay as described above.

Quantification of lipolytic activity

The conditioned media from cell culture treatments and explant cultures were used to quantify the lipolytic activity using the free glycerol assay (Sigma-Aldrich, Saint Louis MO, Cat.# F6428). The levels of free glycerol released indicate the rate of breakdown of triglycerides in the cell. Triglycerides are catabolized to a glycerol and three free fatty acids. Thus, higher levels of free glycerol indicate higher rates of lipolysis. This technique uses a free glycerol reagent that measures the free endogenous glycerol using enzymatic reactions. This technique was performed with a slight modification of the manufacturer's protocol. Briefly, 800 μ L of free glycerol reagent and 100, 200 or 400 μ L of conditioned media were placed in a cuvette to be tested. The volume depended on cell line as basal lipolytic levels varied between the cell lines. A standard curve was generated using a glycerol standard (Sigma-Aldrich, Saint Louis MO, Cat.# G7793). Assay blanks were prepared with glycerol reagent only. The absorbance for each sample was read at 540 nm. The free glycerol levels were determined by comparison to the standard curve. The data was normalized to total cell number (free glycerol levels / total cell number) or tissue weight and compared to untreated as fold over values.

Knockdown of PEDF expression using small interfering RNA (siRNA)

PEDF expression was blocked using siRNA to determine if metformin reduces lipolytic activity and cell proliferation via PEDF in prostate cells. Four different siRNAs directed against PEDF mRNA were tested (Thermo Scientific Dharmacon, Lafayette CO, Cat.# J-010153-05, J-010153-06, J-010153-07, J-010153-08). Lipofectamine (Invitrogen,

Van Allen Way Carlsbad, CA, Cat.# 11668-019) was used as the transfection reagent. The procedure was executed following the general protocol indicated by the manufacturer.

The cells were plated at 15,000 cells/cm² in growth media and incubated at 37°C in 5% CO₂ overnight. The siRNAs were prepared at 5, 10, 50 and 100 nM in order to find the appropriate concentration to block PEDF expression. The siRNA and lipofectamine mixes were incubated at room temperature for 5 minutes, and then the solutions were prepared as indicated in Table 2, mixed, and incubated at RT for 20 minutes. The growth media was aspirated from the cells, and 4.5 mL of fresh growth media without P/S was added. The 500 µL of siRNA-lipofectamine mixture was then added and the cells were incubated at 37°C in 5% CO₂ for 24 hours. After incubation, the transfection media were removed. The cells were washed with PBS and incubated in basal media for 48 h. Cell lysates and conditioned media were collected for analysis of PEDF levels. A non-specific siRNA (Thermo Scientific Dharmacon, Lafayette CO, GAPDH, Cat.# D001810-01) was included as a control for the siRNA transfection.

Table 2. Preparation of siRNA					
siRNA mixture				Lipofectamine mixture	
Concentration siRNA (nM)	Volume siRNA (µL)	Basal media without P/S (µL)		Lipofectamine (µL)	Basal media without P/S (µL)
5	1.25	98.75		6.25	393.75
10	2.5	975.5		12.5	387.5
50	12.5	87.5		62.5	337.5
100	25	75		125	275

Protein Quantification

Protein content in conditioned media and cell lysate samples was determined using a standard Coomassie dye binding assay. The Coomassie reagent and pre-diluted

protein standards were purchased from Thermo Pierce (Rockford, IL, catalog Nos. 1856209 and 23208). The standards were prepared as is indicated by the manufacturer. The samples were either 10 μ l of conditioned media or 5 μ l of cell lysate + 5 μ l of PBS. The samples were added to 490 μ l of Coomassie reagent. Then, the samples were incubated for 5 min at RT. A volume of 200 μ l was placed in duplicate wells on a 96 well plate. The absorbance was read at 595 nm and the concentration was determined from the standard curve.

Determination of PEDF levels by ELISA

PEDF levels were quantified by ELISA following the protocol provided by the manufacturer (Bioproducts MD, Middletown MD, PEDF ELISA kit Cat.# PED613). Briefly, the principle of the assay allows PEDF to bind to an antibody against the PEDF antigen which is bound to the plate. This bound PEDF is detected by a second anti-PEDF antibody conjugated with biotin. Streptavidin peroxidase is then added and this reacts with biotin to produce a color. The optical density is proportional to the amount of PEDF in the sample.

The PEDF antigen standard and all other reagents were prepared according to the manufacturer's directions. Briefly, 20 μ g in a 100 μ L volume of sample per well (conditioned media or cell lysate) and 100 μ l of standards were placed in duplicate wells. The plate was covered and incubated at 37°C for 1 hour. Next, the samples were aspirated, and the plate was washed with 1X wash buffer 5 times with the final wash being aspirated. Next, 100 μ L of PEDF detector antibody was added. The plate was then covered and incubated at 37°C for 1 hour. The samples were aspirated and washed 5

times, and then 100 μ L of streptavidin peroxidase was added. The plate was covered and incubated at 37°C for 30 minutes. Next, the samples were aspirated and washed 5 times. Then, 100 μ L of TMB substrate was added. The plate was incubated at room temperature for 20 minutes, and then 100 μ L of stop solution was added. The absorbance was read at 450 nm using plate reader (Bio-Tek, model KC4, Winooski, VT). The concentration of PEDF in samples was determined by comparison to the standard curve. Values were analyzed as foldover untreated.

Detection of cyclin D1 and AMPK by Western blot

The detection of cyclin D1 (MW=36 kDa) and AMPK (MW=62 kDa) was performed by Western blot using antibodies against phosphorylated AMPK and cyclin D1 (Cell Signaling Technology, Danvers, MA, Cat.# 2535 and 2922, respectively). Cell lysate samples (40 μ g/well) were prepared with 1X Laemmli sample buffer (BioRad, Hercules, CA, Cat.# 161-0737), and were separated on a 12% acrylamide/Bis (29:1) pre-made gel (BioRad, Hercules, CA, Cat.# 456-1043). The samples were boiled for 10 min prior to loading to ensure complete denaturation.

For Western blot hybridization, the membrane was blocked using 50 ml tris-buffered saline (TBS) with 5% powdered milk at RT for 1 hour. The membrane was washed using TBS and then incubated with the first antibody diluted 1:1000 in TBS-Tween (0.1%) (TBS-T) + 5% bovine serum albumin (BSA; Sigma Aldrich, Saint Luis, MO, Cat.# A6003) at 4°C overnight. The membrane was washed with TBS-T three times, 50 ml per wash, and then incubated with the secondary antibody (HRP conjugated) diluted at 1:100-1:5000 in TBS-T + 5% BSA for 1 hour at RT. The membrane was

washed with TBS-T three times, as described above. Next, enhanced chemiluminescence (ECL) detection was performed using Super Signal West Pico chemiluminescent substrate (Thermo Scientific, Rockford, IL, Cat.# 34080) for one minute. Next, the membrane was placed protein side face up in a film cassette and covered with saran wrap. In a dark room, the gel was exposed to X-ray film (Thermo Scientific, Rockford, IL, Cat.#34093) from 1-5 minutes or longer as needed to obtain a useable exposure.

To probe with another antibody, membranes were stripped of antibody. The membrane was washed with TBS-T and a 0.1% solution of SDS was boiled and then poured on the membrane. The membrane was then cooled down to RT and washed again with TBS-T. Finally, the membrane was rehybridized with the next antibody as described above. For a control of protein loading between samples on the gel, membranes were probed with antibodies against GAPDH (Cell Signaling, Danvers MA, Cat.# 7074. This was performed on a stripped blot as described above.

Statistical methods

All the experiments were repeated at least twice. Dependent t-test was used in order to determine changes in cell proliferation (total cell number and viability), PEDF levels, free glycerol levels, and triglyceride levels during metformin with/without OA treatments between each group and the control. ANOVA analysis was used in order to determine dose response in cell culture experiments. All analyses were performed by the statistical analysis software available within the Sigmaplot program (v12.0, Systat Software, Inc, San Jose California). A result was considered significant when $P \leq 0.05$. The data reported were normalized to total cell number or tissue weight and compared to untreated as a fold over values.

RESULTS

The effects of OA with/without metformin on proliferation in prostate cell lines.

The effects of metformin have been previously studied in prostate cell lines. It has been reported that metformin at 5 mM dose reduces cell proliferation in LNCaP, DU145, and PC-3 cells [50]. However, these studies used a high dose of metformin which is not possible to achieve in human patients. One of the main objectives of this research is to test physiologically relevant doses of metformin. The 20 μ M and 0.5 mM doses of metformin are more physiologically relevant doses, and also the 5 mM dose was used in order to compare the results of this research with the results reported in the literature. The use of oleic acid (OA) to overload the cells with lipids is an innovative methodology used to simulate a high fat diet in cancer cells (Doll, *et al*, unpublished data). In my research, I also investigated if metformin blocks OA effects on prostate cell lines and if the PEDF pathway is involved.

The first step was to evaluate metformin's effect on cell proliferation in the presence or absence of lipid overload. RWPE-1, LNCaP, DU145, and PC-3 prostate cell lines were treated for 48 h with metformin at 20 μ M, 0.5 mM, and 5 mM doses with or without OA (1.0 mM). In RWPE-1 cells, OA treatment alone produced a slight, but not significant, reduction in total cell number in comparison with untreated cells (Figure 1A). Metformin treatment alone produced a significant increase in total cell number at 20 μ M ($P=0.016$) and 0.5 mM ($P=0.012$) doses, but, interestingly, metformin reduced the total cell number at the 5 mM ($P=0.048$) dose; however, metformin with OA treatment did not produce any significant changes in comparison with OA alone (Figure 1A). In the viability assay, OA treatment alone reduced cell viability ($P<0.001$) in comparison with

untreated cells (Figure 1B). Metformin treatment alone produced a significant reduction in the 5 mM dose ($P=0.012$), and OA with metformin did not produce significant changes at any dose in comparison with OA treatment alone (Figure 1B). The reduction of total cell number and viability during metformin treatment at the 5 mM dose indicates that metformin treatment of RWPE-1 cells produces a reduction of cell proliferation, possibly by increasing cell death; but, at lower doses, metformin appeared to increase proliferation.

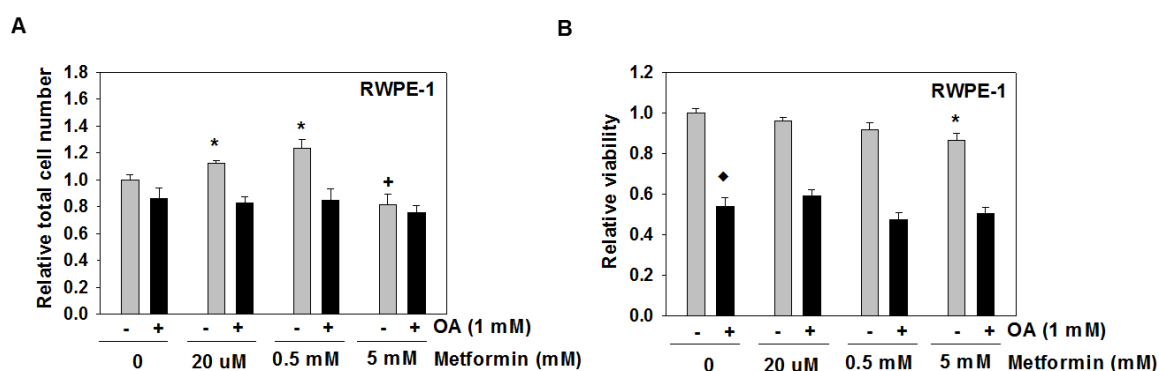


Figure 1. The effects of OA with/without metformin on proliferation and cell viability in RWPE-1 cells. After OA with/without metformin treatments the cells were and incubated with 0.04% trypan blue and (A) total cell number (*significantly increased compared to untreated cells, $P\leq 0.016$; +significantly reduced compared to untreated cells, $P=0.048$) and (B) Viability were assessed (♦significantly decreased compared to untreated cells, $P<0.001$; *significantly decreased compared to untreated cells, $P=0.048$). The results presented are the combined data of three experiments.

In the androgen-sensitive LNCaP cell line, the total cell number results showed that OA treatment alone produced a slight increase in total cell number in comparison with untreated cells which was not statistically significant (Figure 2A). Metformin treatment alone did not produce a significant change in total cell number at any dose (Figure 2A). OA with metformin increased total cell number at 20 μ M ($P=0.037$) and 5 mM ($P<0.001$) in comparison with OA treatment alone (Figure 2A). In the viability assay, OA treatment alone decreased viability ($P<0.001$) in comparison with untreated

cells (Figure 2B). Metformin treatment alone did not produce a significant change at any dose in comparison with untreated cells. OA with metformin significantly reduced viability in a dose-dependent manner (ANOVA, $P=0.002$; Figure 2B). These results suggest that metformin, in presence of OA, reduces viability of LNCaP cells.

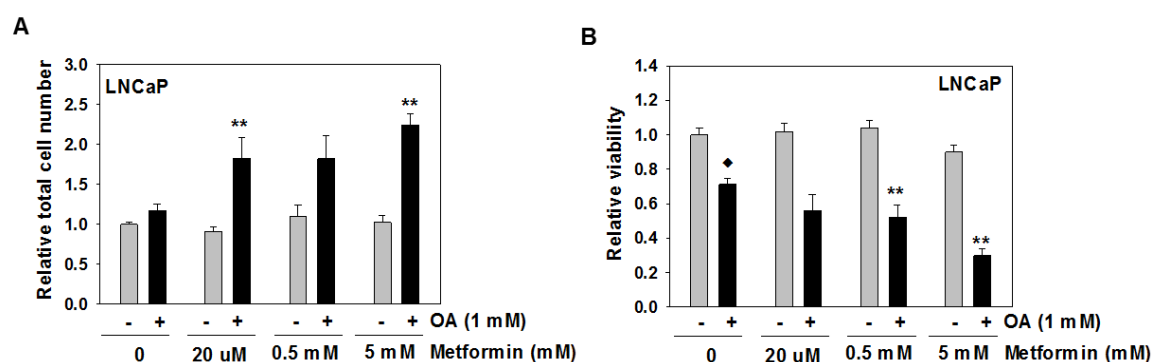


Figure 2. Metformin + OA increase total cell number and reduces viability in LNCaP cells. Total cell number and viability was determined in LNCaP cells after metformin with/without OA treatments. (A) Total cell number was measured by direct cell counts on a Cellometer (**significantly increased compared to OA untreated cells, $P<0.037$). (B) Viability was determined on the Cellometer with trypan blue exclusion assay (♦significantly decreased compared to untreated cells, $P<0.001$; **significantly decreased compared to OA untreated cells, $P<0.034$). The results presented are the combined data of three independent experiments.

In PC-3 cells, OA treatment alone had no effect on total cell number in comparison with untreated cells; however, it significantly reduced viability (Figure 3A). Metformin treatments showed that metformin alone produced a significant decrease in total cell number at 0.5 mM ($P=0.035$) and 5 mM ($P<0.001$) doses in comparison with untreated cells (Figure 3A). In contrast, OA with metformin produced a no significant increase in total cell number at 5 mM ($P=0.059$), in comparison with OA treatment alone (Figure 3A). The results of the viability assay showed that OA alone reduced viability ($P<0.001$) in comparison with untreated cells (Figure 3B). Metformin alone at any dose did not produce significant change in comparison with untreated cells (Figure 3B).

Similarly, metformin in combination with OA did not produce significant changes at any dose in comparison with OA treatment alone (Figure 3B). These results indicate that metformin in PC-3 cells reduces cell proliferation by reducing total cell number while OA decreases viability.

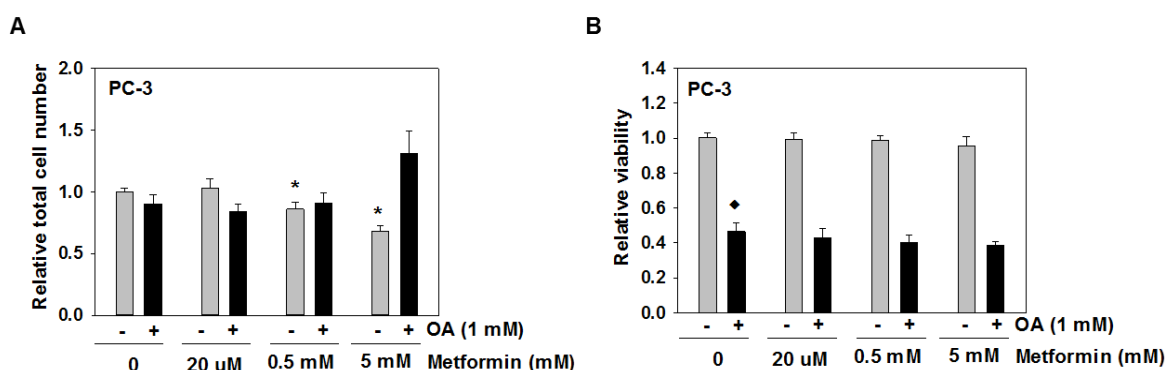


Figure 3. Metformin reduces total cell number and OA decreases viability in PC-3 cells. Total cell number and viability was determined in PC-3 cells after metformin with/without OA treatments. (A) Total cell number was measured by direct cell counts on a Cellometer (*significantly decreased compared to untreated cells, $P < 0.035$). (B) Viability was determined on the Cellometer with trypan blue exclusion assay (♦significantly decreased compared to untreated cells, $P < 0.001$). The results presented are the combination of 2 experiments with similar results.

DU145 was the last cell line in which cell proliferation was evaluated. In the total cell number, OA treatment alone produced a significant increase in comparison with untreated cells ($P < 0.001$), while metformin treatment alone produced a significant reduction in proliferation at the 0.5 mM ($P = 0.008$) and 5 mM ($P = 0.005$) doses in comparison with untreated cells (Figure 4A). The metformin activity demonstrated a dose-dependent response (ANOVA $P = 0.006$; Figure 4A). OA with metformin did not produce significant changes at any dose in comparison with OA treatment alone (Figure 4A). The viability assay showed that OA treatment alone reduced viability significantly in comparison with untreated cells ($P < 0.001$; Figure 4B). Metformin treatment alone produced a significant increase in viability at the 20 μ M dose in comparison with

untreated cells ($P=0.027$). Finally, OA with metformin reduced viability at 5 mM dose in comparison with OA treatment alone ($P=0.006$; Figure 4B). These results indicate that, in DU145 cells, metformin decreases cell proliferation by reducing total cell number since cell viability is not changed, while OA reduced cell viability which was enhanced in the presence of metformin at 5 mM dose.

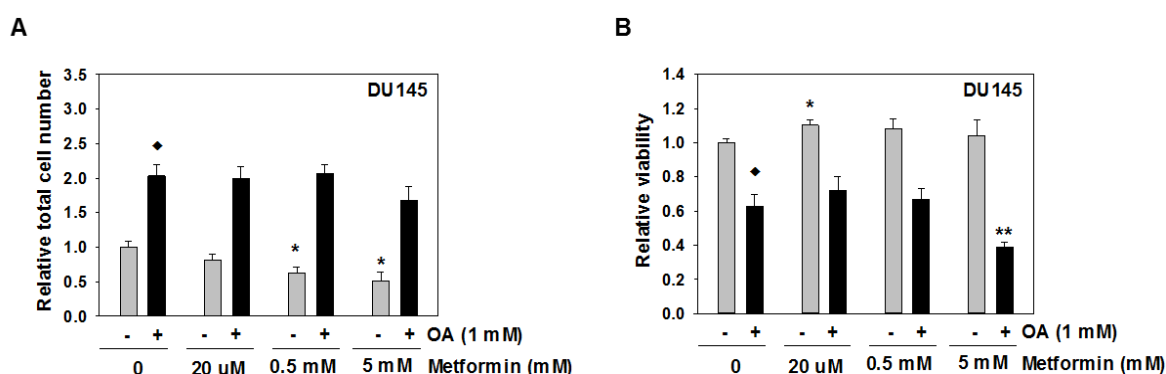


Figure 4. Metformin reduces total cell number and OA reduces viability in DU145 prostate cancer cells. Total cell number and viability was determined in DU145 cells after metformin with/without OA treatments. (A) Total cell number was measured by direct cell counts on a Cellometer (◆significantly increased compared to untreated cells, $P<0.001$; *significantly decreased compared to untreated cells, $P=0.008$). (B) Viability was determined on the Cellometer with trypan blue exclusion assay (◆significantly decreased compared to untreated cells, $P<0.001$; *significantly increased compared to untreated cells, $P=0.027$; **significantly decreased compared to OA untreated cells, $P=0.006$). The results presented are the combined results of two experiments.

The cell proliferation assay results showed that metformin treatment alone produced a reduction in total cell number in RWPE-1, PC-3, and DU145 cells, and reduced viability in RWPE-1 cells. OA treatment alone increased total cell number in DU145 cells, but, in combination with metformin, increased total cell number in LNCaP cells. Otherwise, OA alone or in combination with metformin reduced viability in the four cell lines.

Effects of OA and metformin on lipid metabolism

Lipid accumulation by qualitative assay: The lipid accumulation, produced by the OA treatment, was previously examined using Sudan black B staining in DU145 and PC-3 cells which showed an increase in lipids with OA treatment (Doll, unpublished data). This finding was validated using the neutral lipid-specific stain oil red O (ORO) after OA treatments in all cell lines (Figure 5A-D). In all cell lines, basal levels of lipids were low, and the addition of metformin treatment alone did not alter these levels (Figure 5A-D). In addition, metformin in the presence of OA in RWPE-1, LNCaP, and DU145 cells did not affect lipid accumulation (Figure 5A-C). However, in PC-3 cells, metformin reduced the induced-lipid accumulation in a dose-dependent manner (Figure 5D).

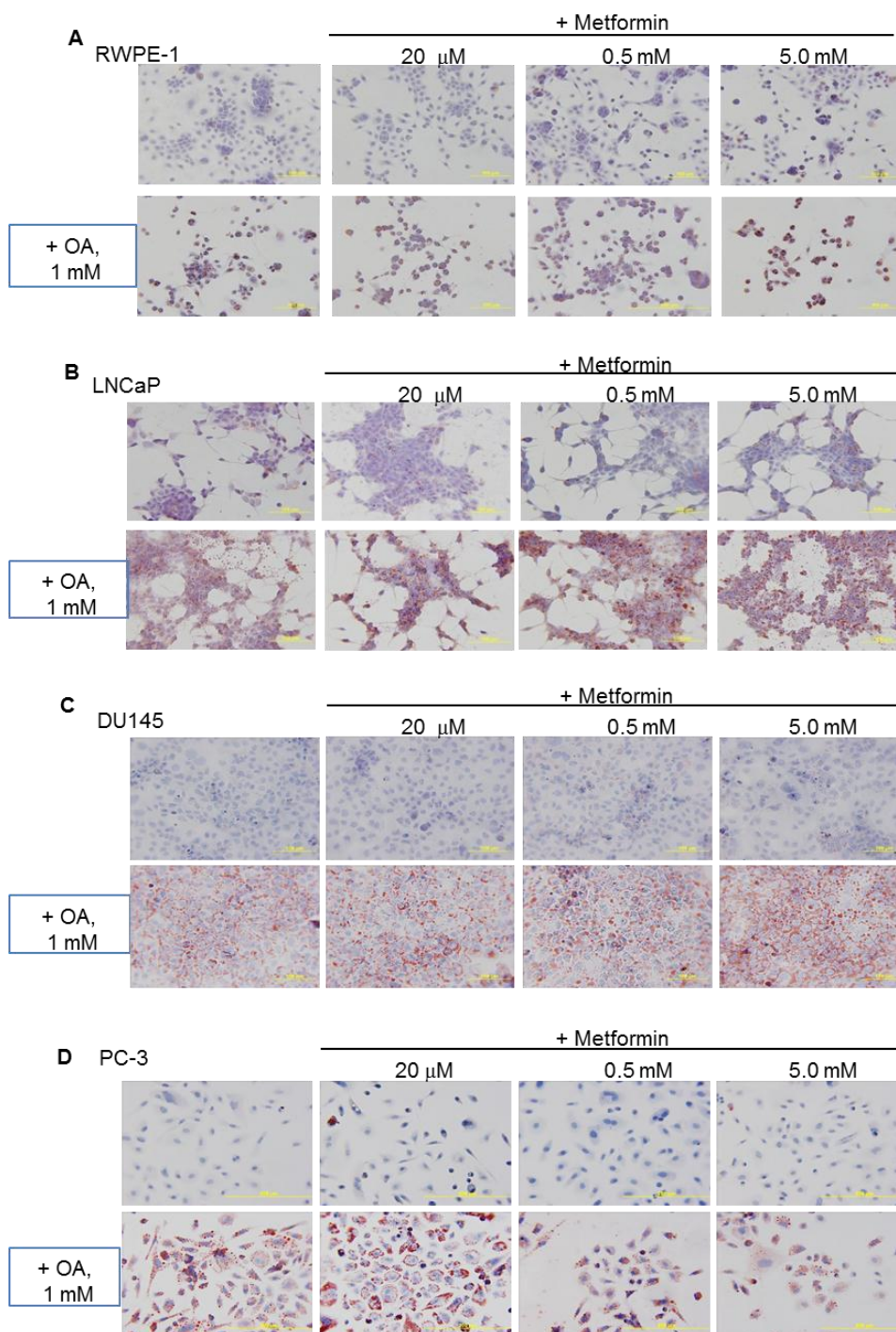


Figure 5. Metformin effect on lipid accumulation. RWPE-1, LNCaP, PC-3, and DU145 cell lines were treated with OA with or without metformin. After treatments, the cells were stained with the neutral lipid-specific stain oil red O (ORO) technique. Lipids are stained red and nuclei are stained pale blue. In all cell lines, OA treatment induced lipid accumulation (A-D). In RWPE-1 (A), LNCaP (B) and DU145 (C) cells, metformin alone at any dose did not block lipid accumulation induced by the OA. (D) In PC-3 cells, metformin reduced lipid accumulation during OA treatment. Each experiment was done twice with similar results.

Lipolytic activity: To evaluate if metformin affected triglyceride catabolism, the lipolytic activity was evaluated by the quantification of free glycerol levels in the conditioned media collected after OA with/without metformin treatments. In RWPE-1 cells, the free glycerol assay showed that OA treatment alone increased lipolytic activity only slightly, and this was not significant, in comparison with untreated cells (Figure 6A; $P=0.108$). Metformin treatment alone produced a significant increment in lipolytic activity at 0.5 mM dose in comparison with untreated cells ($P<0.001$; Figure 6A). Metformin alone, at 5 mM dose, slightly increased the lipolytic activity, but it was not significant ($P=0.111$; Figure 6A). Otherwise, OA with metformin did not produce significant reductions or increases in lipolytic activity at any dose in comparison with OA treatment alone (Figure 6A).

In LNCaP cells, OA treatment alone did not affect the lipolytic activity. However, metformin treatment alone significantly increased lipolytic activity at 0.5 mM and 5 mM doses in comparison with untreated cells ($P\leq 0.001$; Figure 6B). Moreover, there was a significant increase in lipolytic activity during OA treatment with metformin in a dose response manner (ANOVA, $P<0.001$; Figure 6B).

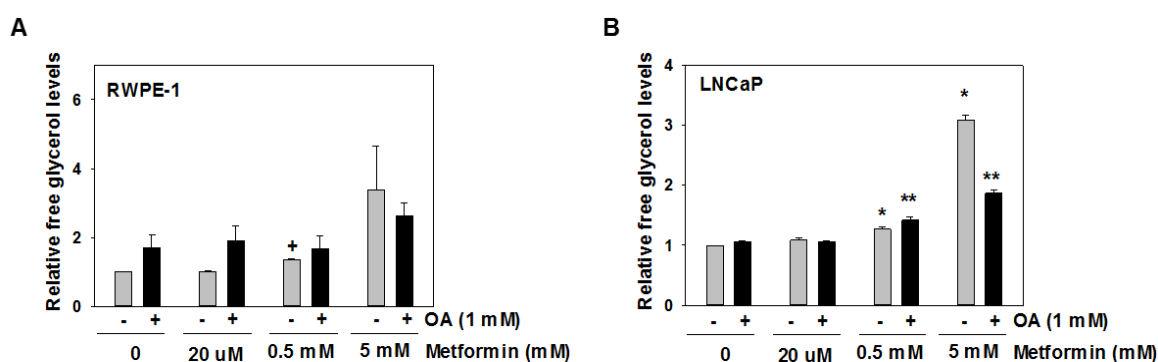


Figure 6. Metformin increases lipolytic activity in RWPE-1 and LNCaP cells. Free glycerol levels were measured in conditioned media after OA with/without metformin treatments in (A) In RWPE-1 cells (\dagger significantly increased compared to untreated cells, $P<0.001$) and (B) in LNCaP cells (\ast significantly increased compared to untreated cells, $P<0.001$; $\ast\ast$ significantly increased compared to OA untreated cells, $P<0.001$). The results presented are the combined data of two independent experiments.

In DU145 cells, interestingly, OA treatment suppressed lipolytic activity ($P<0.001$; Figure 7A). In contrast, metformin treatment alone significantly increased the lipolytic activity at the 20 μ M ($P=0.025$) and at the 0.5 mM ($P=0.027$) doses in comparison to untreated cells (Figure 7A). OA with metformin at 5 mM dose produced a significant increase in the lipolytic activity in comparison with OA treatment alone; however, it was not big enough to overcome the reduction produced by the OA alone (Figure 7A). The intracellular content of triglycerides was also determined in DU145 cells in order to quantitatively evaluate the lipid content. In DU145 cells, as expected, OA treatment alone significantly increased triglyceride levels in comparison with untreated cells ($P=0.025$, Figure 7B). Metformin treatment alone did not significantly change triglyceride levels at any dose, in comparison with untreated cells (Figure 7B). In contrast, metformin at 0.5 and 5 mM doses, in the presence of OA, reduced triglyceride levels in comparison with OA alone; however, it did not reach significance at any dose (Figure 7B).

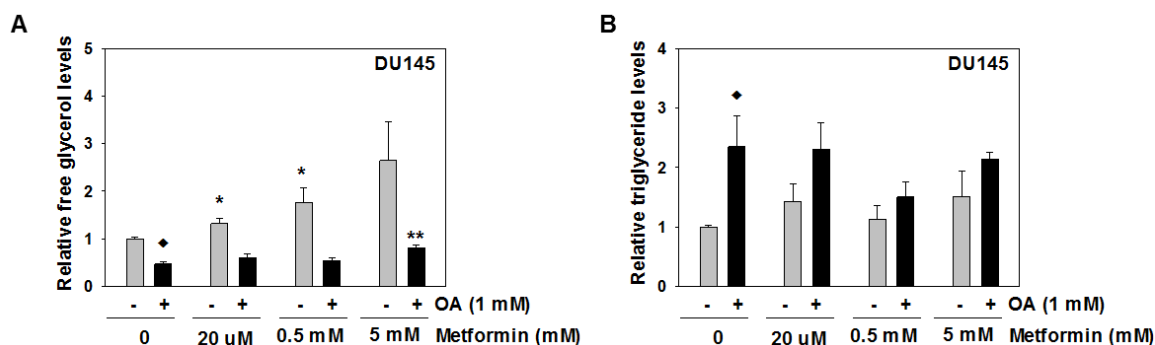


Figure 7: Metformin increases lipolytic activity in DU145 cells. (A) Free glycerol levels were measured in conditioned media after metformin with/without OA treatment (♦significantly decreased compared to untreated, $P<0.001$; *significantly increased compared to untreated, $P<0.027$; **significantly increased compared to OA untreated, $P=0.003$). (B) Triglyceride levels were measured in cell lysates after metformin with/without OA. Triglyceride levels (♦significantly increased compared to untreated, $P=0.025$). The results presented in each graph are the combined results of two experiments

In contrast to DU145 cells, in PC-3 cells, OA stimulated lipolysis ($P<0.001$) in comparison with untreated cells (Figure 8A). Moreover, metformin treatment alone did not produce a significant change in lipolytic activity at any dose (Figure 8A). Also, metformin did not alter the effect of OA on lipolytic activity (Figure 8A). As expected, when triglyceride levels were determined, OA alone increased triglyceride levels in PC-3 cells ($P=0.025$; Figure 8B). Interestingly, in PC-3 cells, the metformin treatment alone significantly increased triglyceride content at 0.5 mM and 5 mM doses in comparison with untreated cells ($P<0.001$); but, OA with metformin reduced triglyceride levels in a dose manner response (ANOVA, $P<0.001$; Figure 8B). This observation is consistent with the reduction in the lipid accumulation observed in the ORO assay (Figure 5D). These results suggest that metformin blocks lipid accumulation in PC-3 cells by reducing the lipid uptake. In summary, the observation that all prostate cancer cell lines responded differently to both the OA and metformin treatment is very interesting. It suggests that

genetic differences between the cell lines likely have a significant impact on the functional pathways affected by these treatments.

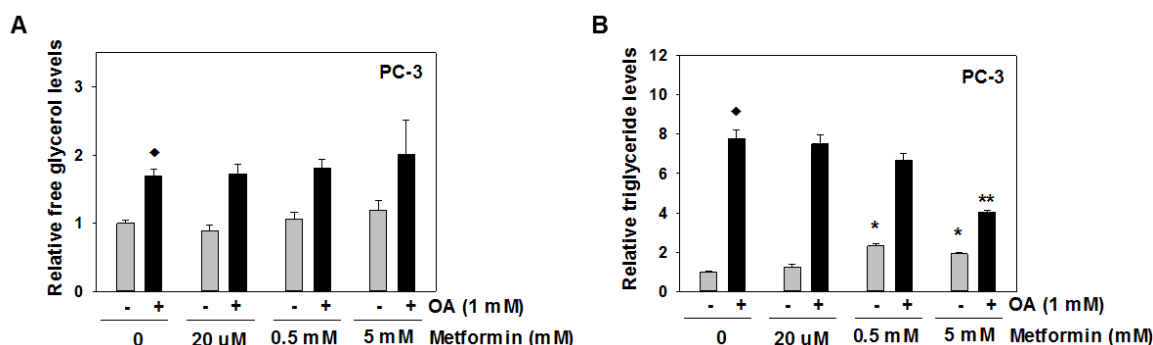


Figure 8. Metformin reduces triglyceride levels in PC-3 cells. (A) Free glycerol levels were measured in conditioned media after metformin with/without OA treatment (♦significantly increased compared to untreated cells, $P<0.001$). (B) Triglyceride levels were measured in cell lysates after metformin with/without OA (♦significantly increased compared to untreated cells, $P<0.001$; *significantly increased compared to untreated cells, $P<0.001$; **significantly decreased compared to OA untreated cells, $P<0.001$). The results presented are the combined results of two experiments.

Determination of PEDF levels during OA with or without metformin treatments

In order to determine if metformin regulates PEDF levels, the levels of PEDF were measured in cell lysates and conditioned media collected from the four cell lines after OA with/without metformin treatments. Both the lysate and conditioned media were examined because, while PEDF has primarily been studied as a secreted protein, there is also a cytoplasmic pool of PEDF. Not surprisingly, based on the data obtained thus far, results varied by cell line as well as between the conditioned media and cell lysate for each line.

In the cell lysates of RWPE-1 and PC-3 cells, metformin treatment alone at any dose did not produce significant change in PEDF levels (Figure 9A-B). In DU145 cells metformin produced a dual effect, it produced a significant reduction of PEDF levels at the 20 μ M dose ($P=0.025$), and a non-significant increase at the 5 mM dose ($P=0.270$) in

comparison with untreated cells (Figure 9C). In LNCaP cells, metformin treatment alone increased PEDF levels at the 5 mM ($P<0.001$) dose in comparison with untreated cells (Figure 9D). OA treatment alone did not produce significant change in PEDF levels in RWPE-1, PC-3, and LNCaP cells (Figure 9A,B,D). However, in DU145 cells, OA treatment decreased PEDF levels significantly in comparison with untreated cells ($P=0.007$; Figure 9C). OA with metformin did not affect PEDF levels in any cell line at any dose in comparison with OA treatment alone (Figure 9A-D).

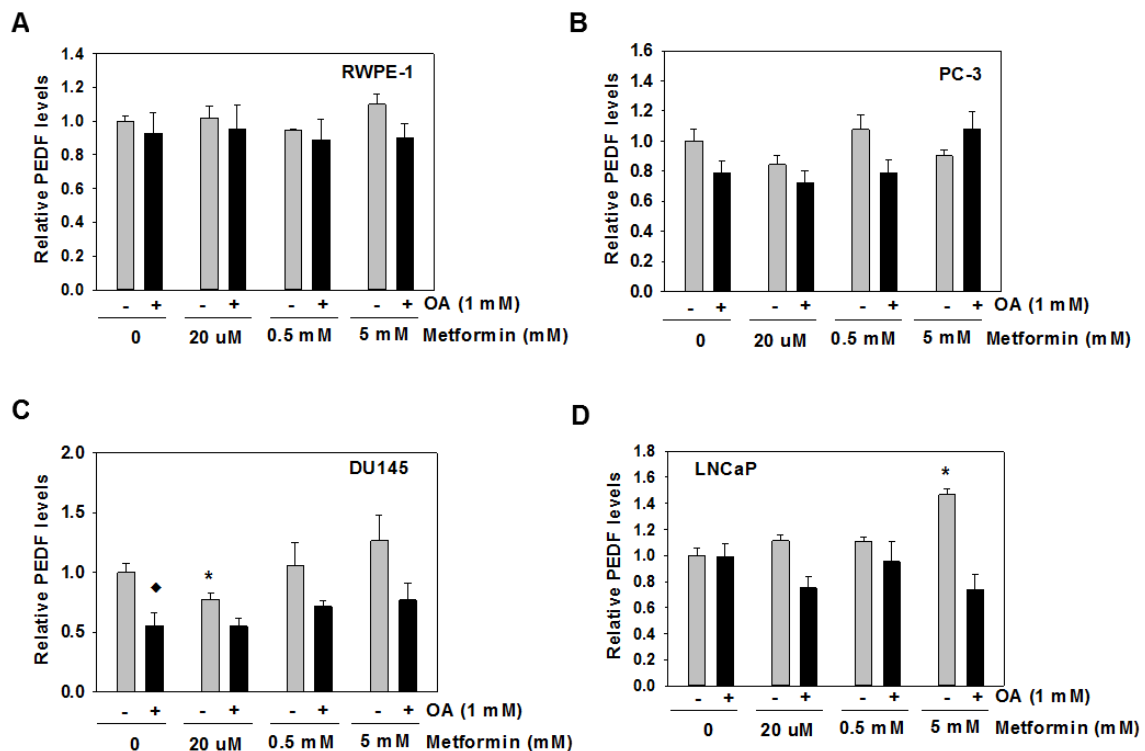


Figure 9. Metformin with or without OA effect on intracellular PEDF levels. After metformin with/without OA treatments, cell lysates were collected and PEDF levels were measured by ELISA. In RWPE-1 (A) and PC-3 (B), metformin with/without OA treatments did not produce significant changes at any dose. In DU145 cells (C), metformin and OA significantly reduced PEDF levels (*Significantly decreased compared to untreated, $P=0.025$; ♦significantly decreased compared to untreated, $P=0.007$). In LNCaP cells (D), metformin treatment alone increased PEDF levels (*Significantly increased compared to untreated, $P<0.001$). The results presented are the combined data of two independent experiments.

In striking contrast to the variable results seen in the cell lysates, assays of the conditioned media showed that OA consistently suppressed secreted PEDF levels in all cell lines ($P < 0.05$; Figure 10A-D). In RWPE-1 and LNCaP cells, metformin treatment alone, at the 0.5 mM dose, induced a significant increase in PEDF levels in comparison with untreated cells ($P \leq 0.003$, Figure 10A,C). Otherwise, metformin induced a reduction in PEDF levels in RWPE-1 cells at the 5 mM ($P < 0.001$) dose and in PC-3 cells at the 0.5 mM ($P = 0.010$) dose in comparison with untreated cells (Figure 10A,B). OA with metformin did not produce a significant change in PEDF levels in any cell line in comparison with OA treatment alone (Figure 10A-D). These results suggest that OA may selectively regulate secreted PEDF levels versus intracellular PEDF.

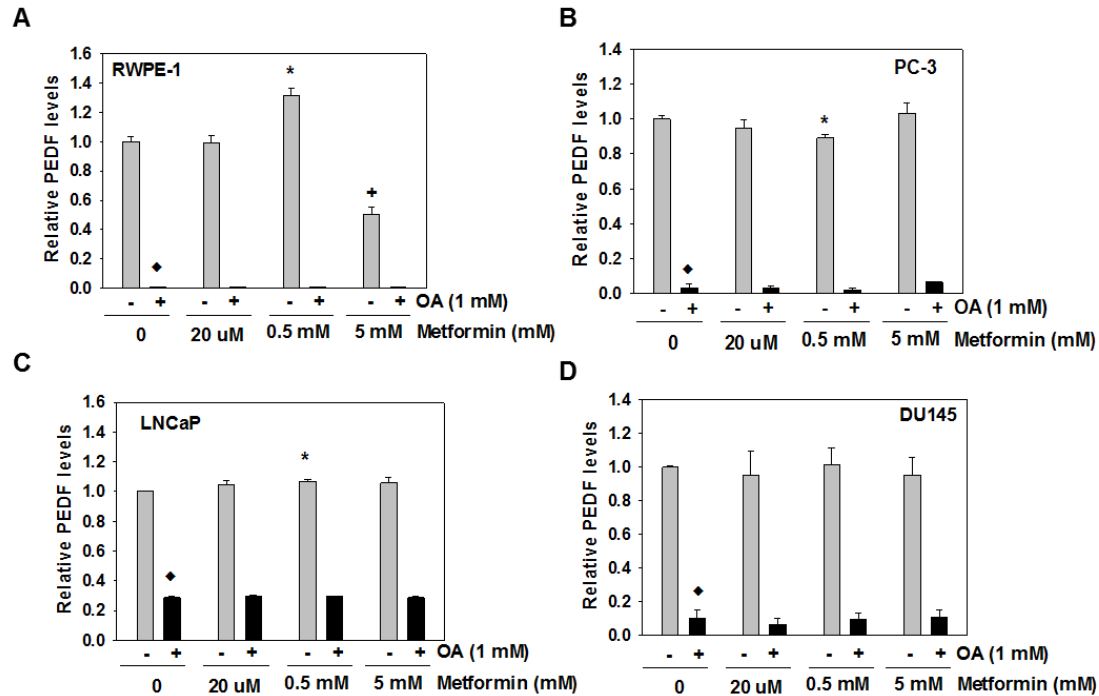


Figure 10. OA treatment suppressed secreted PEDF levels while metformin had little impact. After metformin with/without OA treatment the conditioned media were collected and PEDF levels were measured by ELISA. OA reduced PEDF levels in the four cell lines. Metformin alone increased PEDF levels in RWPE-1 and LNCaP cells. Metformin with OA did not produce significant changes at any dose in comparison with untreated. (A) RWPE-1 cells (*Significantly increased compared to untreated, $P=0.002$; + significantly reduced compared to untreated, $P<0.001$; ♦significantly reduced compared to untreated, $P<0.001$). (B) PC-3 cells (*Significantly increased compared to untreated, $P=0.010$; ♦significantly reduced compared to untreated, $P<0.001$). (C) LNCaP cells (*Significantly increased compared to untreated, $P=0.003$; ♦significantly reduced compared to untreated, $P<0.001$). (D) DU145 cells (♦Significantly reduced compared to untreated, $P<0.001$). Metformin increased extracellular PEDF levels in RWPE (A) and LNCaP (C) cells. The results presented are the combination of two independent experiments

Anti-PEDF siRNA treatment to block PEDF expression

The next step, in order to establish that PEDF plays a direct role in metformin-response in prostate cells, would be to block PEDF expression and determine if the response to metformin is lost. The DU145 cell line was used to test anti-PEDF siRNAs. Four different anti-PEDF siRNA (PEDF 5, 6, 7, and 8) at 3 different doses (10, 50, and 100 nM) were used and mixed with lipofectamin to facilitate the transfection. After treatment with the siRNAs, the conditioned media and cell lysates were collected, and

PEDF levels were measured by ELISA. The non-targeting control siRNA had no effect on PEDF levels in either the cell lysates or conditioned media. In conditioned media, the anti-PEDF siRNA number 8 presented the best inhibition of PEDF at 50 nM ($P=0.007$) and 100 nM ($P=0.010$) doses in comparison with untreated cells (Figure 11A). However, the inhibition was only 40% compared to untreated cells, and it is recommended that at least 80% inhibition be achieved in order to continue any test with siRNAs. In the cell lysates, there was no significant PEDF inhibition with any of the anti-PEDF siRNA at any dose (Figure 11B). Thus, further optimization or different anti-PEDF siRNAs are needed to inhibit PEDF sufficiently. Interestingly, these observations suggest that there may be different mRNAs that are transcribed to produce the two different pools of PEDF. Also of interest, the regulation by the siRNA was similar to OA treatment, where secreted PEDF levels were suppressed while cellular PEDF levels were not. Together, these data suggest that PEDF regulation may be more complex than originally expected.

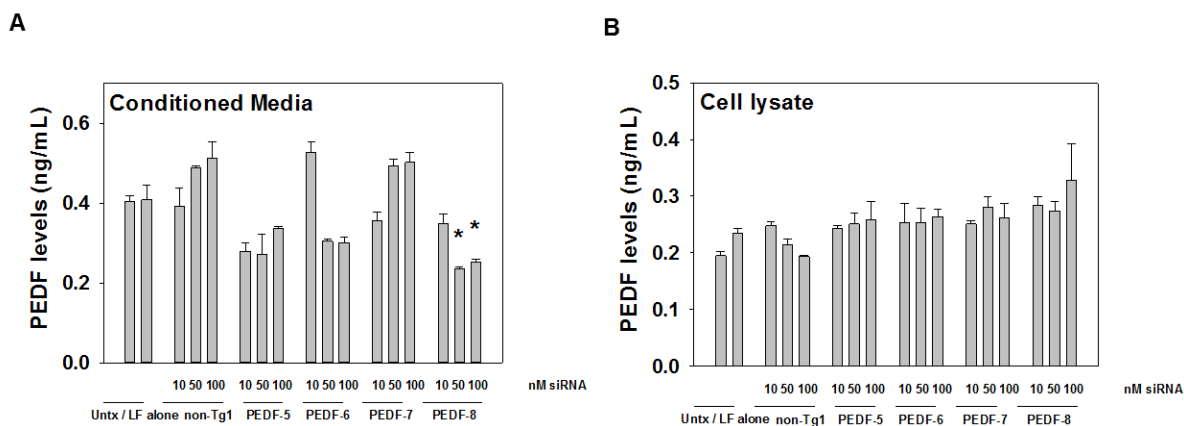


Figure 11. Use of anti-PEDF siRNA inhibits PEDF expression in DU145 cells. Four different anti-PEDF siRNA were tested for inhibition of PEDF expression, PEDF-5, -6, -7, and -8. (A) In conditioned media the anti-PEDF siRNA #8 presented the biggest inhibition (*Significantly reduced compared to untreated, 50 nM $P=0.007$, 100 nM, $P=0.010$). (B) In cell lysates none of the anti-PEDF siRNA produced inhibition compared to untreated. The results presented are the combined results of two experiments.

Lipolytic activity and triglyceride content in wild type versus PEDF KO prostate tissue

In order to extrapolate the research carried out in the *in vitro* experiments to *in vivo* activity, the lipolytic activity and the triglyceride content were determined *ex vivo* in wild type and PEDF KO prostate tissues. The dorsolateral left prostate of wild type and PEDF KO mice (n=5 per group) were used to measure the free glycerol levels. The PEDF KO mice presented with a reduced lipolytic activity in comparison with the wild type tissues; however, this was not statistically significant ($P=0.153$; Figure 12). Based on the trend of decreased lipolytic activity observed in the PEDF KO tissues, future studies should analyze additional mice to determine if a true difference exists. In addition, all lobes of the mouse prostate should be analyzed to establish if there is a difference between lobes.

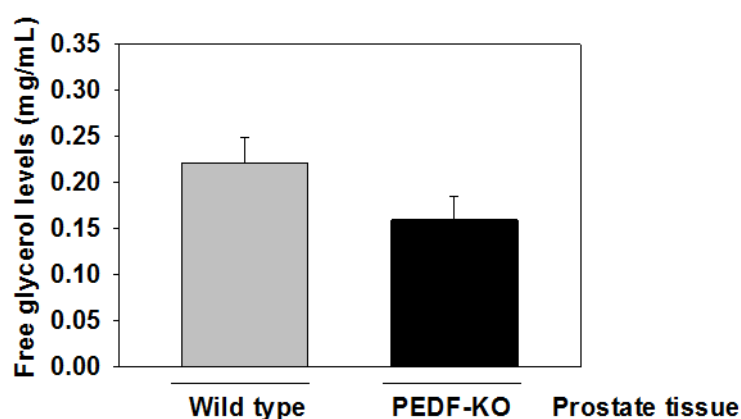


Figure 12. Loss of PEDF may reduce lipolytic activity in prostate tissue. Free glycerol levels were measured in dorsolateral prostate tissues (left lobe) collected from wild type and PEDF KO mice (n=5 each group). Tissues were minced for explant culture and lipolytic activity was measured in the serum-free conditioned media using the free glycerol assay. The difference between the wild type and PEDF KO activity was not significant ($P=0.153$).

Triglyceride levels were quantified in the ventral, dorsolateral right lobe, and anterior prostate lobes of wild type and PEDF KO mice (n=5 per group). Values were normalized per gram of weight of tissue, and, interestingly, each prostate lobe showed different triglyceride content (Figure 13). The ventral prostate of the PEDF KO mice presented higher levels of triglycerides in comparison with the wild type tissues. In the dorsolateral right lobe, PEDF KO mice had a slightly reduced triglyceride content in comparison to wild type tissues, while in the anterior prostate lobe, triglyceride levels were similar in both mouse strains (Figure 13). While the data obtained in the mouse studies were not significant, the trends observed suggest that further studies, to increase the number of animals analyzed, are warranted to determine if true differences exist.

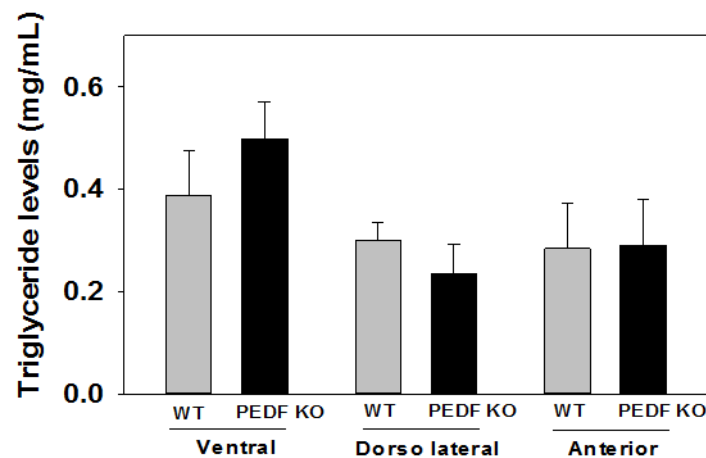


Figure 13. Effect of PEDF loss on triglyceride content in prostate tissues. Triglyceride levels were measured in wild type (WT) and PEDF KO prostate tissues. PEDF KO ventral prostate presented higher content of triglycerides in comparison with WT ($P=0.356$). PEDF KO-dorsolateral right prostate presented a lower content of triglycerides than WT ($P=0.345$). PEDF KO and WT anterior prostate presented similar triglyceride levels ($P=0.690$).

DISCUSSION

The purpose of this thesis was to determine if the anticancer effect of metformin is mediated in part via PEDF. Metformin is a drug used by diabetic patients to control their sugar levels. Several studies have shown that diabetic patients on metformin present a reduction in incidence and progression of different cancers, including prostate cancer [35, 36, 39, 41]. Also, it has been reported that metformin reduces cell proliferation in LNCaP, DU145, and PC-3 prostate cell lines [50]. The mechanism of action of metformin has been studied on prostate cancer cells, however, its mechanism of action remains unclear. It is possible that in prostate cancer cells, different pathways are involved. For example, in LNCaP and DU145 cells metformin blocked G_0/G_1 phase, with a reduction of cyclin D1, to produce a reduction in cell proliferation or viability [50]. In other cancer cell types, activation of AMPK has been demonstrated [39, 40, 43]. However, in prostate cancer cells metformin induction of AMPK did not seem critical [50]. Metformin could also increase lipolysis and reduce lipogenesis, affecting the lipid stores that are needed for cellular processes.

I hypothesized that PEDF may mediate metformin's activity in prostate cancer cells. A published study showed that diabetic PCa patients on metformin presented with higher serum PEDF levels in comparison with non-diabetic patients not on metformin [51]. Moreover, a previous experiment found, using immunohistochemistry, that metformin increased PEDF levels in prostate cancer cells (Doll, unpublished data). The results of my experiments thesis showed that metformin produced different effects among the prostate cell lines used, depending on if it was administrated alone or in combination with OA. In general, metformin treatments showed a decrease in cell proliferation,

increase in lipolytic activity. Also, metformin induced a slight increase in PEDF levels of RWPE-1 and LNCaP cells. These results suggest that a signal pathway for metformin activity is in part through PEDF expression. Therefore, the following specific aims were studied to support my hypothesis.

Specific aim 1. Elucidate if metformin mediates its anti-proliferative activity through PEDF in the presence or absence of lipid overload by:

- a) Quantifying PEDF level in metformin with or without OA treated prostate cells;
- b) Assessing levels of cyclin D1 and activated AMPK in metformin with/without OA treated cells; and
- c) Establishing if anti-PEDF siRNA treatment blocks metformin's anti-proliferative effect.

The first step was to evaluate the effect of several doses of metformin on cell proliferation and viability in prostate cancer cells (LNCaP, DU145, and PC-3) and a normal prostate epithelial cell line (RWPE-1). In a study by Sahra, *et al*, it was reported that metformin reduced cell proliferation by approximately 50% in LNCaP, DU145, and PC-3 cells [50]. The authors tested metformin at 5 mM dose and 3 days later the cell proliferation was determined using the MTT assay. That study presents the drawback that the authors used a high dose of metformin which is not physiologically possible to reach in humans. The actual doses of metformin that are recommended in diabetic patients are 500-2000 mg per day; thus, the highest plasma concentration would be 10.8 μ M (NDA 20-357/S-031, NDA 21-202/S-016). In addition, the authors assessed proliferation using

the MTT assay, a metabolic assay which functions as an indirect indicator of cell proliferation. The MTT assay is based on the ability of living cells to convert the MTT reagent into formazan crystals. This works as an indicator of mitochondrial activity which is directly related with the number of living cells [56]. However, it is recommended to use direct cell counting to determine live cells together with the MTT assay because metformin is known to increase the activity of the mitochondria to increase the fatty acid oxidation [43]. Thus, the MTT could not be accurate. The cell proliferation experiments presented here were performed using direct cell counts with trypan blue exclusion assay to obtain total cell numbers and viability data.

The cell proliferation data showed that metformin treatment alone significantly reduced total cell number in RWPE-1 (at 5 mM dose), PC-3 (at 0.5 and 5 mM doses), and in DU145 (at 0.5 and 5 mM doses) cells. Moreover, metformin treatment alone did not reduce viability in LNCaP, PC-3, or DU145 cells, at 20 μ M, 0.5 mM, or 5 mM doses. However, there was a reduction in viability in the RWPE-1 cells with metformin at the 5 mM dose. The differences in the results between cell lines in my data and between my results and published studies [48-50] could result from several factors. In the published studies, the authors plated fewer cells/cm² and assayed after three days of treatment with 1 mM metformin. Thus, this high dose of metformin on the cell lines for three days might reduce cell proliferation more than the two days treatment that I performed. Also, the MTT assay was carried out in 96 well plates; thus, the use of a smaller plating surface combined with using a metabolic assay may also account for the contrasting results as well since 10 cm dishes were used in my studies.

One likely explanation for the differences that I observed in response to treatment between cell lines in my studies is the genetic background of the cell lines. Many studies have described genetic differences among the prostate cancer cells lines commonly used in prostate cancer research studies [57-62]. The presence or absence of different gene products within each of the cell lines during treatment could be the basis of the observed differences in the results. Table 3 indicates some of the key genetic differences present among prostate cell lines. For example, p53 and Rb genes control cellular senescence. Interestingly, the four cell lines present different patterns of expression in these two genes. Moreover, my cell proliferation data showed that each cell line presented a different response after metformin and OA treatments. Based on this information, the differences in the expression of p53 and Rb could, in part, explain the differences observed in the cell proliferation assays. Similarly, the differences in the expression of tumor suppressor genes and oncogenes among the cell lines could also impact the lipid metabolism, such as the lipolytic activity, in each cell line.

Table 3. Genes commonly altered in the prostate cell lines used in this study.*				
Gene name	RWPE-1	LNCaP	DU145	PC-3
<i>Tumor suppressor genes</i>				
PTEN	Wildtype	Mutant	Wildtype	Null
P53	Null	Wildtype	Mutant	Null
Rb	Null	Wildtype	Mutant	Wildtype
<i>Oncogenes</i>				
Bcl-2	Wildtype	Wildtype	Null	Wildtype
c-myc	Null	Null	Wildtype	Wildtype

Key: Wildtype, produces a normal, functional protein; Null, produces no protein; Mutant, produces a non-functional protein.

**Information based on [57-62]*

It has been shown that OA treatment increases cellular proliferation in DU145 and PC-3 cells lines using MTT assays and direct cell counts with trypan blue exclusion (Doll, unpublished data). However, my data showed that OA treatment alone

significantly increased total cell number just in DU145 cells, and OA with metformin significantly increased total cell number in LNCaP cells. A consistent finding was that OA reduced viability in the four cell lines. Similarly, OA with metformin further reduced the viability in LNCaP and DU145 cells. Thus, my results show that metformin reduces cell proliferation by reducing total cell number or viability depending if is administrated alone or in combination with OA.

The Ben Sahra, *et al*, study showed that cyclin D1 was decreased in DU145 cells, but not in PC-3 cells, after metformin treatment at 5 mM [50]. To examine this in my samples, I performed Western blots of DU145 and PC-3 cells. These showed that the expression of cyclin D1 is still present at 20 μ M and 0.5 mM doses of metformin, and the reduction of cyclin D 1 was only observed at 5 mM dose (data not shown). While this does correlate with the published study, it also suggests that during the expression of cyclin D1 at 20 μ M and 0.5 mM doses of metformin alternative mechanisms are involved to reduce cell proliferation. A caveat to this, however, is that a blot with a house keeping gene to normalize the cyclin D1 expression was not obtained; thus, the studies have to be repeated before a conclusion can be reached.

Another potential mechanism to explain the antiproliferative effect of metformin is the activation of AMPK. Metformin has been shown to activate AMPK in hepatocytes which decreased acetyl CoA carboxylase and increased fatty acid oxidation [43]. This activity produces an overall reduction in the fatty acid stores available in the cell. Ben Sahra, *et al*, found that metformin at 5 mM dose produces an upregulation of AMPK in LNCaP, DU145, and PC-3 cells [50]. Moreover, they found that the inhibition of AMPK did not affect the anti-proliferative effect of metformin at the 5 mM dose [50]. Thus, the

authors indicated that AMPK was not necessary for the metformin effect in the prostate cell lines studied. To see if this can be applied to lower doses of metformin, the AMPK activation was examined among the samples in. My Western blot data showed that AMPK was activated by metformin in at the 0.5 and 5 mM doses in DU145 and PC-3 cells (data not shown); however, at the 20 μ M dose, there appears to be a decrease of AMPK. Again, however, these observations have the caveat that normalization to a housekeeping gene was not done. So, while no firm conclusions can be drawn, these data are consistent with my observations of triglyceride levels in the cells. For example, in PC-3 cells the increased expression of AMPK during metformin with OA treatment is consistent with the reduction in the triglyceride levels. Also, the upregulation of AMPK is consistent with the increase in the lipolytic activity in DU145 cells. This suggests that this lipid regulatory function could participate in the reduction of cell proliferation. To investigate this, future experiments would need to block lipolytic activity or to overexpress cyclin D1 to determine which ameliorates metformin's activity.

The experiments showed that metformin produced an increase in intracellular PEDF levels in RWPE-1 and LNCaP cells. Moreover, the OA treatment reduced extracellular PEDF in all four of the cell lines tested, and the addition of metformin did not overcome the effect of OA. In addition, the fact that OA reduces extracellular PEDF, but does not reduce intracellular PEDF, suggests that there may be different mechanisms regulating the two PEDF pools such as the production of two different mRNAs from the PEDF gene. In fact, it has been reported that different isoforms of PEDF are produced, each one with different properties [63]. Thus, it is possible that each PEDF isoform participates in different cellular processes. For example one could participate in lipid

metabolism activities such as the reduction of lipid accumulation (PC-3 cells), or lipolytic activity (RWPE-1, LNCaP, and DU145 cells). Another PEDF isoform might participate in the regulation of cell proliferation. Thus, future experiments to specifically compare intra- and extra-cellular PEDF, as well as the PEDF mRNAs produced in the prostate cell lines are needed.

In summary, the results for Aim 1 showed that: 1) PEDF is present in both the cytoplasmic and secreted protein pools; 2) that metformin slightly increased PEDF levels at different doses; and, 3) that OA reduced PEDF levels in the conditioned media of all four cell lines. Thus, it is possible to propose that metformin has some stimulatory activity on PEDF levels, but that this effect was not sufficient to overcome OA suppression. Thus, a higher PEDF concentration might be needed in order to overcome changes in the lipid metabolism in the presence of lipid overload. Also, it seems possible that the reduction of secreted PEDF levels produced by OA is related to the reduction in lipolytic activity. Therefore, studies blocking PEDF during metformin and OA treatment are needed.

In this point of my investigation, the inhibition of PEDF during metformin treatment would test if PEDF participates in metformin's activity and the regulation of cyclin D1 and AMPK in prostate cells. In an attempt to do this, I tested four different anti-PEDF siRNAs. DU145 cell line was selected because it showed reduction in cell proliferation and active lipolytic activity during metformin with/without OA treatments. The use of the anti-PEDF siRNA #8 in DU145 cells showed inhibition of 40% in comparison with untreated cells. However, at least an 80% inhibition is required to

continue any cellular siRNA assay. Thus, future experiments are necessary to optimize this assay and determine if the participation of PEDF is required for metformin's activity.

Specific aim 2. Examine if metformin regulates lipid metabolism in prostate cancer cells by:

- a) Assessing lipid levels qualitatively (ORO staining) and quantitatively (direct triglyceride quantification) in metformin treated cells with and without OA treatment;
- b) Quantifying the lipolytic activity in samples from 2a; and,
- c) Examining the levels of lipolytic activity in explant cultures of prostate tissues from wild type versus PEDF knockout mice and quantify triglyceride levels in these tissues.

The effects of metformin on lipid metabolism (Specific aim 2a,b) has not been studied in prostate cells. The first step to evaluate the role of metformin on lipid metabolism was by the ORO assay after metformin with or without OA treatments. In my experiments, metformin did not reduce lipid accumulation in RWPE-1, LNCaP, and DU145 cells, but it did reduce lipid accumulation in PC-3 cells. It has been reported that metformin treatment reduced lipid accumulation in macrophages due to a reduction of the expression of fatty acid-binding protein 4 (FABP4) which participates in lipid uptake [46]. This protein is also expressed on PC-3 cells [47], thus the result obtained in the ORO assays suggest that this protein may participates in the lipid metabolism of PC-3 cells. Thus, future studies are needed to investigate the role of this protein in the regulation of lipid uptake in PC-3 cells.

It has been reported that metformin activates AMPK in the hepatocytes which regulates lipid metabolism [43]. This AMPK activation increases fatty acid oxidation, and a necessary step toward this, also induces lipolytic activity to produce free fatty acids from triglycerides. The results for the specific aim 2b showed that OA alone produced a significant reduction in lipolytic activity in DU145 cells and an increase in PC-3 cells. Metformin treatment alone increased lipolytic activity in RWPE-1, LNCaP, and DU145 cells. Metformin in the presence of OA increased the lipolytic activity in RWPE-1, LNCaP, and DU145 cells. These results show that the genetic differences among the cell lines (discussed above) affect different pathways during metformin with or without OA treatment. Thus, microarrays analysis of genes that are up- or down-regulated with metformin treatments in the presence or absence of OA, would facilitate understanding of how the genetic background of the cell lines affect response to this treatment.

Specific aim 2c was successfully carried out using prostate tissue of PEDF KO mice. I anticipated that loss of PEDF would reduce lipolytic activity, and I did observe a reduction in the dorsolateral prostate lobe although this did not reach statistical significance. This result was consistent with the results obtained *in vitro*. Based on the lipolytic activity in PEDF KO tissue, I expected an increase in the triglyceride levels in the PEDF KO prostate tissues. However, just the PEDF KO ventral tissue presented with an increase in triglyceride levels, although this also was not statistically significant. The trends observed support the idea that PEDF regulates lipid metabolism in the prostate, but additional tissues need to be examined in order to determine if the differences are significant.

CONCLUSION

The hypothesis of this investigation was that the antiproliferative effect of metformin is in part mediated via PEDF. Two specific aims were addressed in order to test the hypothesis. The specific aim #1 was to establish if the antiproliferative effect of metformin was via PEDF in the presence or absence of lipid overload. The first set of experiments tested the antiproliferative effect of metformin on prostate cell lines. In these experiments physiologically relevant doses of metformin (20 μ M and 0.5 mM) were tested, which until now has not been reported in the literature. Moreover, the testing of the effect of metformin in presence of lipid overload is also novel. My cell proliferation data contrasted with previous published studies that indicate that metformin reduces cell proliferation at 5 mM dose in LNCaP, DU145, and PC-3 cells [50]. My data showed that metformin alone at 5 mM dose significantly reduced the viability in just RWPE-1 cells. However, metformin alone reduced total cell number in PC-3 and DU145 cells. OA alone reduced viability in the 4 cell lines. Metformin with OA reduced the viability in LNCaP and DU145 cells, which suggests that in these cell lines, some pathways are activated to reduce cell proliferation but possibly they are not activated in RWPE-1 and PC-3 cells. Thus, my data showed that metformin possesses an antiproliferative effect that consists of a reduction in total cell number and viability, and the reduction of viability is enhanced in the presence of lipid overload. Due to technical difficulties with the siRNA inhibition of PEDF, this sub-aim was not completed. Thus, future studies will seek to optimize siRNA experiments to achieve PEDF suppression.

Specific aim 2 was focused on determining if metformin regulates lipid metabolism in prostate cancer cells and if so, if this activity is mediated via PEDF. These

studies are the first to examine the effects of lipid overload and metformin on prostate cancer cells. The data showed that metformin increased lipolytic activity at different levels depending on the cell line, dose, and if it was administered alone or in combination with OA. Metformin treatment alone increased lipolytic activity in RWPE-1, LNCaP, and DU145 cells. Metformin in combination with OA increased lipolytic activity in LNCaP and DU145 cells. Also, it is important to point out that metformin, in the presence of OA, reduced triglyceride levels in PC-3 cells, which is consistent with the reduction in lipid accumulation observed in this cell line.

Based on these data, it was clear that metformin and OA treatments have an impact on cell proliferation and lipid metabolism in prostate epithelial cells. The measurement of intracellular and secreted PEDF levels during metformin with or without OA treatments showed that PEDF is affected modestly by metformin, but that OA has a more significant impact. Therefore, the reduction of PEDF during OA treatments may impact metformin's effect. Thus, future experiments blocking PEDF expression are critical to evaluate its role during metformin treatments. As an alternative to evaluate the effect of PEDF inhibition, I used the PEDF KO mouse prostate tissues as an *ex vivo* model. The PEDF KO prostate tissues had somewhat reduced lipolytic activity in comparison with WT. Moreover, the lack of PEDF affected the triglyceride levels among the prostate lobes. However, these results were not statistically significant and additional animals are needed to establish a real effect.

In summary, the work presented here supports a novel molecular pathway to explain the antiproliferative effect of metformin on prostate cells. My data support my

hypothesis that PEDF is involved in the metformin pathway. In addition, my data demonstrate that metformin also regulates lipid metabolism in prostate epithelial cells.

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