

**REGULATION OF ANTI-ANGIOGENIC PEDF THROUGH A
TSP-1 – CD36 PATHWAY IN PROSTATE CANCER**

by

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ABSTRACT

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Prostate cancer (PCa) is the most common type of cancer diagnosed in American men. Cancer progression is associated with increased angiogenesis, and thrombospondin-1 (TSP-1) and pigment epithelium derived factor (PEDF), both potent anti-angiogenic molecules, are downregulated in PCa cells. TSP-1 exerts its activity through binding to several cell surface receptors such as CD36. Both TSP-1 and PEDF are multi-functional proteins and have been linked to lipid metabolism in other cell types. Moreover, TSP-1 – PEDF regulatory loops have been identified in some cell types. PEDF has been shown to inhibit PCa growth through its effects on angiogenesis and directly on the PCa cells; however, how PEDF expression levels are regulated in prostate cells is currently unknown. Here, we hypothesized that TSP-1 may regulate PEDF expression and lipid metabolism in PCa cells.

I collected and examined PEDF levels in both cell lysate and serum-free conditioned media samples from TSP-1 and anti-CD36 antibody treated prostate cells and examined PEDF levels. Both TSP-1 and anti-CD36 treatment increased PEDF expression in normal prostate epithelial cells, RWPE-1, and in PCa cells, PC-3 and LNCaP. The expression of candidate TSP-1 – CD36 signaling mediators, fyn, p38 MAPK and JNK, were also examined in treated samples. I found that TSP-1 treatment elevated expression of fyn, p38 and JNK in PC-3 and DU145 cells. In

contrast, blocking the CD36 receptor diminished the expression of each signaling mediator.

My results are the first to show that a regulatory loop exists between TSP-1 and PEDF in prostate cells. The observation that treatment with anti-CD36 antibody also increased PEDF suggests that TSP-1 regulation of PEDF may be mediated through the CD36 receptor. These observations suggest that one mechanism of PEDF down-regulation in PCa cells may be due to loss of TSP-1 expression. Moreover, this pathway could be exploited for novel therapeutic interventions.

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INTRODUCTION

Prostate cancer

Prostate cancer (PCa) is the most commonly diagnosed type of cancer and the second leading cause of death in men in the United States [1]. As estimated by the National Cancer Institute at the National Institutes of Health, there will be 238,590 new cases and 29,720 deaths due to prostate cancer in 2013 [1]. It is estimated that by the age of 80, approximately 80% of men will have some cancerous cells in their prostate [2]. PCa screening is performed by measuring the serum prostate specific antigen (PSA) in the blood in conjunction with a digital rectal examination (DRE). If these tests indicate that PCa may be present, a transrectal ultrasound (TRUS) guided needle biopsy is taken for histologic examination for a definitive diagnosis. Prostate carcinomas are assigned a Gleason score to indicate histological grade. The Gleason grading system is the dominant grading system used for prostate carcinomas worldwide [3]. It is based on the histology of the carcinoma cells of the prostate. A Gleason score ranges from 2-10, with scores over 7 considered high grade patterns.

While these approaches have led to increased diagnosis of cancer in men who may not have a clinically significant disease [4], the chance of cancer-specific survival is improved for intermediate and high risk cancers [5]. There are several treatment options for PCa including androgen deprivation therapy, surgical removal of the prostate and radiation therapy [6]. Androgen deprivation therapy is often the first line of treatment for metastatic cancers; however, most men eventually progress to hormone refractory disease. Chemotherapy can provide a survival benefit for some men with hormone refractory, metastatic disease; however, new treatments are needed.

Prostate cancer, diet and obesity

Despite much work, the causes of PCa have not yet been well elucidated, although some risk factors, such as age, race, genetic allelic variants, and family history have been identified [7]. Globally, the incidence and mortality of prostate cancer vary widely [1]. Diet is associated with prostate cancer risk and mortality as established in epidemiological studies [7]. It has been reported that diet may have a greater impact on the aggressive form of disease as compared to the indolent form of PCa [8]. Epidemiological studies suggests that lycopene, from cooked tomatoes, may be protective against developing PCa; however, these data have not been entirely consistent (as reviewed in [9]). Epidemiological data suggests that there is a high correlation between PCa risk and dietary fat derived from meat (as reviewed in [9]). Consumption of red meat, particularly, has been consistently linked to development of more aggressive disease [10-14]. While, there is no correlation between obesity and PCa risk [8], studies have suggested that obese men are diagnosed more often with more advanced and high grade cancer [8]. Obese men are also more likely to die of PCa than lean men [8, 15]. Serum androgen levels and other potential PCa growth factors have been shown to be influenced by body mass. Thus, while obesity is not associated with increased PCa risk, it makes the disease worse. However, the association between the two, as established by epidemiological studies, has been inconsistent [16-20].

Role of angiogenesis inhibitors in PCa

Like most solid tumors, the growth of PCa beyond a few millimeters in diameter is dependent on induction of angiogenesis, the growth of new vessels from existing vessels [21]. Complex interactions between vascular growth factors, including both positive and negative factors, regulate the growth of new blood vessels [22-24].

There is a fine balance between high levels of inhibitors and low levels of inducers in most normal tissues [25, 26]. However, when inducer secretion predominates, the endothelial cells in the adjacent vessels are stimulated to proliferate and migrate toward the source tissue [27]. The increase of inducers or decrease in the secretion of inhibitors or a combination of both occurs as result of genetic or epigenetic alterations in cancers [25, 28]. Decreased expression of several angiogenic inhibitors occurs in PCa, including decreased expression of thrombospondin-1 (TSP-1) and pigment epithelium derived factor (PEDF) [29, 30]. Both of these proteins have multiple, cell type specific functions.

PEDF in PCa

Pigment epithelium derived factor (PEDF) is a 50kDa secreted glycoprotein that belongs to the serpin (serine protease inhibitors) group [31]. PEDF was first discovered as a differentiation factor for retinoblastoma cells [32, 33]. It was isolated from the conditioned media of the retinal pigment epithelial cells. PEDF levels are known to decline with age and it serves as a marker for young cells [34, 35].

PEDF is a potent inhibitor of both physiological and pathological angiogenesis [36]. Downregulation of PEDF has been observed in many cancer types, such as melanoma, cervical cancer and PCa [37-39]. Also, the decreased expression of PEDF has been associated with poor prognosis and increased metastasis in the prostate [30]. It has been reported that re-expression of the PEDF gene in human hormone refractory PCa PC-3 cells is associated with decreased tumor growth [40]. Recently, Hirsch *et al.* showed that PEDF suppresses IL-8 production in PCa cells [41]. IL-8 has been shown to be involved in PCa progression. In mice, loss of PEDF expression is associated with the development of prostatic epithelial cell hyperplasia and increased stromal vascularization in the prostate [30].

PEDF is also secreted by adipocyte and is involved with insulin resistance in the body [42, 43]. It has been shown to have a profound effect on lipid metabolism. Studies have shown that PEDF influences systemic fatty acid metabolism by promoting lipolysis in an adipocyte triglyceride lipase dependent manner [44]. However, whether PEDF, lipid metabolism and PCa are linked remains to be explored.

TSP-1 in PCa

Thrombospondins are a family of five multidomain, calcium binding extracellular glycoproteins. They are synthesized and secreted in a wide variety of cells [45]. Amongst all the members of the thrombospondin family (TSP-1, TSP-2, TSP-3 TSP-4 and TSP-5), TSP-1 has been studied most widely. It was the first naturally occurring inhibitor of angiogenesis to be identified [46]. TSP-1 is a large (450kDa) trimeric molecule that binds to several receptors and ligands. It serves as a prototype member of the family [46, 47]. TSP-1 was first discovered in activated platelets [48]. A variety of functions are associated with the TSP-1 molecule. It stimulates matrix assembly by binding to matrix proteins such as collagen and fibronectin and also regulates matrix digestion by metalloproteinases and plasmin. TSP-1 has been shown to stimulate apoptosis of the endothelial cells and T cells; however, it promotes survival of the vascular smooth muscle cells [49, 50]. Fibroblasts, smooth muscle cells, adipocytes and macrophages are amongst a few cells that secrete TSP-1 [51, 52]. TSP-1 is a major activator of transforming growth factor- β (TGF- β) and also activates neutrophils [53-55]. In the prostate, activation of TGF- β by TSP-1 plays a crucial role in regulating prostate growth [56]. The expression of TSP-1 is regulated by the tumor suppressor p53 in prostate tissue [57]. One of the ways by which TSP-1 inhibits angiogenesis is that it causes apoptosis in macrophages,

eventually reducing inflammation and attenuating cytokines responsible for angiogenesis [58].

Tumor progression is a multistep process wherein the cells acquire (1) the ability to evade apoptosis, (2) insensitivity to antigrowth signals, (3) self-sufficiency in growth signals, (4) limitless replicative potential, (5) sustained angiogenesis and (6) the ability to invade and metastasize [59]. TSP-1 is important in regulating angiogenesis. The expression of TSP-1 is decreased in many cancer types, including prostate, pancreas and urothelial cancer [29, 60, 61]. A decrease in TSP-1 expression in prostate carcinoma cells has been associated with mutant p53 [57]. Naturally occurring angiogenesis inducers, such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF-2), are increased in PCa tissues [29]. This promotes tumor progression as a result of sustained angiogenesis. In PCa, TSP-1 has been thought to suppress angiogenesis by creating a matrix barrier between tumor and stroma [62]. Tissue levels of TSP-1 have been found to increase following castration in rat model [63] and in persons undergoing androgen-deprivation therapy [56]. The TSP-1 knockout phenotype has been established to have many phenotypes, including hyperplasia in the prostate [53].

TSP-1 and CD36

TSP-1 binds to several cellular receptors, such as CD36, CD47 and several integrins [64]. CD36 is a single chain, 88 kDa, highly glycosylated protein that is a multi-ligand scavenger receptor involved in fatty acid and lipid metabolism [65]. Amongst the many CD36 functions, it is a transporter of long chain fatty acids and therefore, is also called fatty acid translocase [66]. CD36 is involved in fat and fat soluble vitamin absorption in the liver [67]. In endothelial cells, one study showed that TSP-1 mediated its anti-angiogenic activity through CD36 binding and induction of apoptotic

signals [68]. While CD36 is expressed in the normal prostate and in PCa [69], the interaction between TSP-1 and CD36 in prostate cells has not been studied.

TSP-1 and signaling pathways

In endothelial cells, the anti-angiogenic activity of TSP-1, upon binding with the CD36 receptor is mediated through intracellular signaling molecules [68]. Upon TSP-1 binding with the CD36 receptor, a signaling cascade is initiated. This signaling pathway in endothelial cells involves the non-receptor protein tyrosine kinase Fyn, the mitogen activated protein kinases (MAPKs) p38 and c-Jun N-terminal kinase (JNK), and caspase-3 [68]. It is believed that the CD36 receptor itself does not have any kinase activity and that its oligomers most likely utilize adaptor proteins such as paxillin to initiate signaling (as reviewed in [70]). Src related kinases, such as Fyn [68], are brought into physical proximity by CD36 dimerization caused by TSP-1 [71]. The cascade continues when p38 kinases are activated with subsequent activation of caspases 3 and 7 [68]. This leads to the transcriptional activation of the *fasL*, which serves as a ligand for the extrinsic death receptor CD95/Fas. This signaling pathway is summarized in Figure 1. As signaling pathway of TSP-1 in PCa cells has not been elucidated, the pathway in endothelial cells serves as a model for this study.

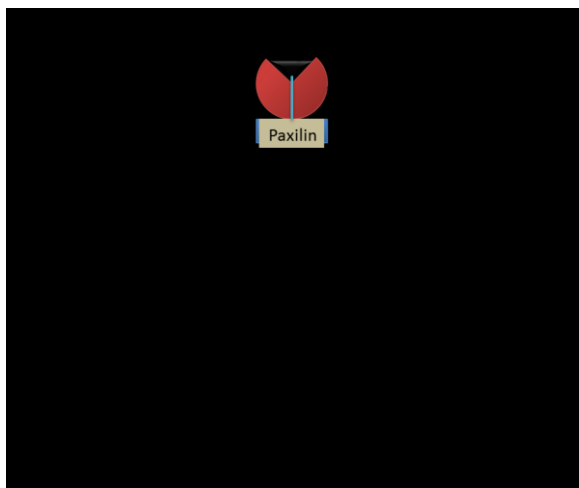


Figure 1. Mechanisms of TSP-1 and CD36 signaling cascade in activated endothelial cells. TSP-1 binding to CD36 stimulates CD36 dimerization which then recruits the adaptor molecule paxilin to initiate a signaling cascade. TSP-1 binding to CD36 brings the src-kinase fyn to physical proximity to CD36, allowing activation. Activated fyn, in turn, phosphorylates (activates) both p38 MAPK and JNK. Kinases p38 and JNK pathways both ultimately lead to induction of apoptosis.

TSP-1 and PEDF interactions in other tissues

Another molecule implicated in TSP-1 signaling is PEDF. In a study by Schmitz et al, PEDF suppresses TSP-1 levels *in vitro* in pancreatic cells [72]. Wang *et al.* observed that TSP-1 levels were reduced in vitreous fluid of diabetics and that this decrease was highly associated with an increase in a high molecular weight PEDF isoform [73]. PEDF has been reported to upregulate TSP-1 production in glioma cells. Guan *et al.* found that glioma cells (U251) overexpressing PEDF showed a 5.3 fold increase in TSP-1 protein with a downregulation in VEGF and basic fibroblast growth factor levels [74]. Consistent with the results in the glioblastoma cell line, Jia and Waxman found that KM12/PEDF-expressing colon tumors displayed an increase in TSP-1 levels [75]. In endometrial cancer cell lines, PEDF induced an increase in the TSP-1 mRNA levels [76]. However, whether TSP-1 influences PEDF expression in PCa is unknown.

Hypothesis and specific aims

PCa is the second most common type of cancer in American men and one of the leading causes of cancer-related death worldwide [1]. The expression of thrombospondin (TSP)-1, a multifunctional glycoprotein protein, is decreased in many cancer types, including PCa [29]. TSP-1 is a matricellular glycoprotein discovered first in activated platelets [48]. While it is a potent angiogenesis inhibitor, it also has many other functions, such as activating transforming growth factor- β [53]. In many cancer types, TSP-1 is downregulated by oncogenes such as c-fos, c-jun and Ras [77, 78]. In PCa cells, the tissue levels of TSP-1 are repressed by androgens [63]. In addition, TSP-1 knockout mice develop prostate hyperplasia, a precursor lesion to cancer, demonstrating the importance of this protein in maintaining the normal prostate [56].

TSP-1 can exert its activity through binding to CD36 [79]. The CD36 receptor plays an important role in fatty acid and glucose metabolism and is also known as fatty acid translocase [65, 80, 81]. The CD36 signaling pathway is mediated via src and MAP kinase family of proteins, including fyn, p38 and JNK in other cell types [68]. While CD36 has been shown to be overexpressed in PCa, its function is unknown. Recent studies have shown that lipid accumulation stimulates proliferation of PCa cells (Doll lab, unpublished observation); however, the mechanism of lipid uptake in these cells is not established. Thus, initially, I hypothesized that TSP-1 and CD36 regulated cellular lipid uptake. In PCa cells treated with oleic acid to induce lipid accumulation, neither TSP-1 nor anti-CD36 antibody treatment affected lipid accumulation, as assessed by Oil Red O (ORO) staining experiments (Figure 2). However, I did find that treatment with either TSP-1 or neutralizing anti-CD36 antibody increased the expression of PEDF. PEDF is another potent anti-angiogenic molecule, which is also downregulated in PCa cells. In other cell types, a regulatory loop between TSP-1 and PEDF has been established; however, the regulation of either protein in prostate cells is unclear. PEDF has been shown to inhibit PCa growth through its effects on angiogenesis and directly on the PCa cells, including induction of lipolysis [30]. Increasing PEDF expression, with TSP-1 treatment and/or neutralizing CD36 treatment, could be a novel target for antitumor therapy in PCa. ***Thus, I hypothesized that TSP-1 binding to CD36 induced PEDF and anti-angiogenic activity. To test this hypothesis, I conducted the following experiments:***

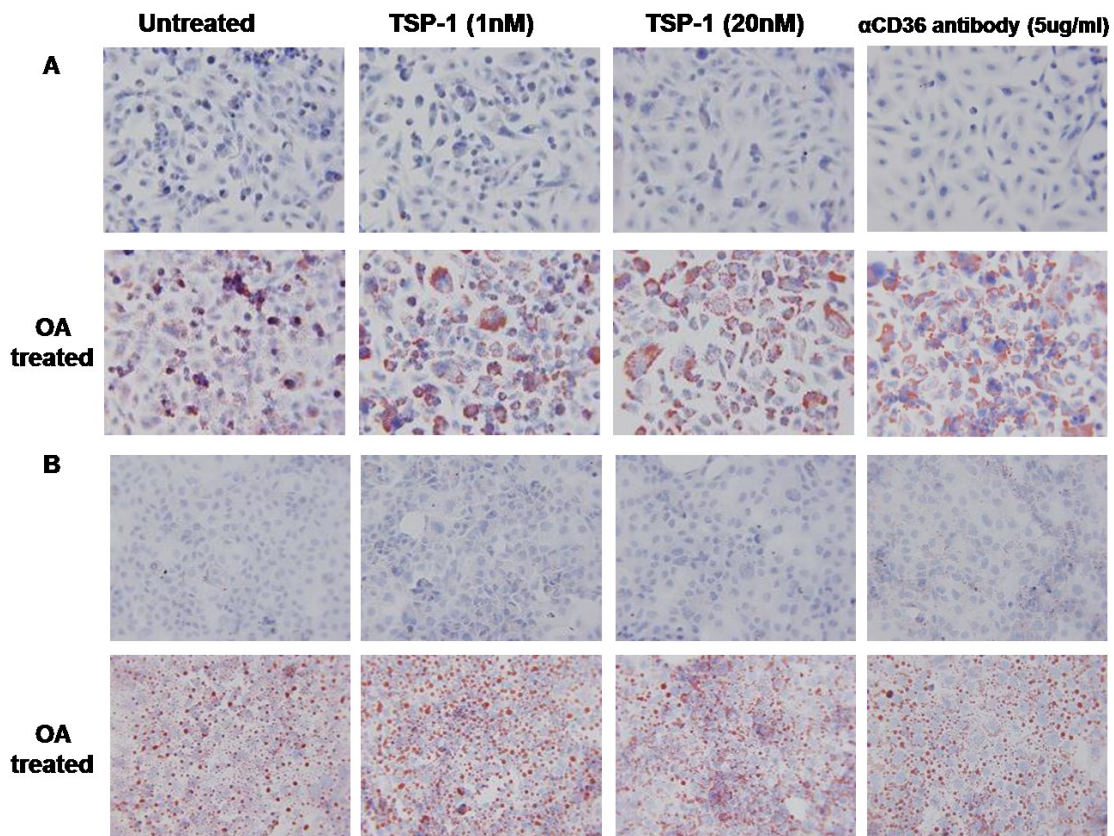


Figure 2. TSP-1 and anti-CD36 antibody treatment did not change cellular lipid accumulation in PC-3 or DU145 cells. PC-3 (A) or DU145 (B) cells were plated on chamber slides and left overnight to allow cell attachment. Cells were then treated with TSP-1 (1, 20 nM) or +/- oleic acid (1 mM) or neutralizing anti-CD36 antibody (5 µg/ml). After 48h, media was aspirated and cells were stained with Oil red O, a neutral lipid stain.

Specific aim 1: To establish that TSP-1 induces PEDF expression through inhibition of CD36 by (a) determining if PEDF expression increases in PCa cells in presence of TSP-1 or anti CD36 antibody treatment; (b) assessing if PEDF levels increase in the presence of anti-CD36 siRNA treatment; and, c) comparing the levels of PEDF expression in prostate tissues taken from TSP-1 and CD36 knockout mice to that of wildtype mice.

Specific aim 2: To elucidate the signaling pathway through which TSP-1 and/or CD36 regulate PEDF expression and determine if the TSP-1-induced PEDF produces a more anti-angiogenic phenotype by (a) Examining the expression levels

of known TSP-1 and CD36 signal mediators fyn, p38 and JNK; and, b). Evaluating changes in secreted angiogenic activity in PCa cells treated in aim 1 above.

Through the experiments proposed here, I will begin to establish the signaling mechanism through which PEDF expression is regulated. This pathway could be a novel target for future PCa therapy development.

MATERIALS AND METHODS

Cells lines and strains

PCa cell lines PC-3, DU145, LNCaP and RWPE-1, the normal prostate epithelial cell strain, and human microvascular endothelial cells (HMVECs) were used in these experiments. PC-3, DU145, LNCaP and RWPE-1 cells were purchased from the ATCC (Manassas, Virginia). All the PCa cell lines were originally isolated from metastatic lesions. DU145 and PC-3 cells are androgen refractory while LNCaP is an androgen sensitive cell line [82]. PC-3 and DU145 cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Sigma, St. Louis, MO, cat# D5796) with 10% Fetal Bovine Serum (FBS, Sigma, St. Louis, MO, cat# F2442), and 1% penicillin / streptomycin (P/S, Cellgro, Manassas, VA, cat # 30-002-CI). LNCaP cells were grown in Roswell Park Memorial Institute Medium (RPMI, Sigma, St. Louis, MO, cat# RNBC4302) media with 10% FBS and 1% P/S. RWPE-1 cells were grown in keratinocyte growth media (Life Technologies, Grand Island, NY cat# 10724-011) with 1% P/S. HMVECs were cultured in gelatinized tissue culture flasks. Flasks were gelatinized overnight at 4°C by incubating the flask's growth surface in a 0.01% solution of gelatin (Difco, Sparks, MD, cat# 214340) in phosphate buffered saline (PBS, Cellgro, Manassas, VA, cat# 21-031-CV) to enhance cell adherence. HMVECs were grown in endothelial cell growth media (EGM, Lonza, Walkersville, MD, cat# CC-4147). All cells were maintained, and all cell experiments were conducted, at 37°C in 5% CO₂.

Cell culture and treatments

For TSP-1 and anti-CD36 (α CD36) antibody treatment studies, cells were plated at 20,000 cells / cm² in 10 cm tissue culture dishes with growth media (day 1). These cells were left overnight to allow cell attachment. Experimental group

media contained either TSP-1 (R&D systems, Minneapolis, MN, cat# 3074-TH) at a dose of 1, 5, 10 or 20 nM or α CD36 antibody (Novus Biologicals, Littleton, CO, cat# NBP1-60151) at a dose of 2 or 5 μ g/ml. On day two, growth media were aspirated, cells were rinsed with PBS, and then experimental treatment media were added. Basal media alone served as a negative control. The cells were then incubated with treatment media for 48 hours. At the end of the 48h treatment, cells were collected for proliferation analysis, cell lysate collection and conditioned media collection. All cell culture experiments were repeated at least two times.

Conditioned Media Collection

Conditioned media from treatment groups was collected for PEDF expression analysis and for functional studies (described below). Conditioned media was carefully pipetted from the tissue culture dishes into conical tubes on ice. These tubes were centrifuged at 800 - 1000 x g for 8 minutes at 4°C to pellet any cellular debris. After centrifugation, conditioned media was transferred to a new conical tube. Aliquots of each sample were then taken for the lipolysis assay (described below). Then, 1X protease inhibitor cocktail (Sigma, St. Louis, MO, cat# P8340) and 1 μ M phenylmethylsulfonyl chloride (PMSF, Sigma, St. Louis, MO, cat# 93482) were added to the conditioned media. To capture floating cells, the tubes containing the cell pellets were then used to collect cells for the proliferation assay (described below).

The protein concentration in the conditioned media of these cell lines is low (Doll *et al*, unpublished data). Therefore, after collection, conditioned media were concentrated using Millipore ultra-15 centrifugal filter device with a 3 kDa cutoff value and 15 ml volume (Millipore, Billerica, MA, cat# UFC9). The centrifugal filter was filled with PBS and centrifuged (4000 x g) at 4°C for 10 minutes to wet the membrane.

The remaining PBS was discarded and the conditioned media was added to the membrane. This was centrifuged (4000 x g) at 4°C for 5-60 minutes, depending on the sample volume and speed of filtration, as the membrane filter cannot be allowed to become absolutely dry. The filtrate was discarded after each spin. Additional conditioned media was added and centrifuged until the entire sample was included. Then, PBS was added up to 2 times the conditioned media volume to wash out the phenol red. Samples were then transferred to a siliconized tube and stored at -80°C until use.

Proliferation assay

Cells were trypsinized (1 ml trypsin 10 cm per dish; Trypsin-EDTA, Cellgro, Manassas, VA, cat# 25-053-CL) and detached cells were added to growth media (3 ml) was used to stop the trypsinization in the tube preserved above. A 50 µl aliquot of this cell solution was added to a tube containing 50 µl of 0.4% trypan blue stock solution (Sigma, St. Louis, MO, cat# T8154). After 5 minute incubation a 20 µl aliquot was added onto a Cellometer cell counting chamber slide (Nexcelom, Lawrence, MA, cat# SD100). A total cell and live cell count, with viability calculation, was performed on the Cellometer (Cellometer Auto T4, Bioscience, Nexcelom, Lawrence, MA) per the manufacturer's instructions. Reported numbers included the dilution factor of 2 for the dilution in trypan blue. Final total counts were calculated by multiplying the calculated number of cells per milliliter by the total volume of cells (4 ml). Counts for each treatment group were performed in duplicate and compared to the negative control.

Cell lysate collection

The cells collected above were pelleted by centrifugation (800 x g for 8 minutes), and the media was aspirated. Cell lysis buffer (1x, Cell Signaling, Danvers, MA, cat# 9803S), prepared with 1x protease inhibitor cocktail and 1 μ M PMSF, was added to the pellet. Cells were mixed, and the solution was transferred to a microfuge tube and incubated on ice for 5 minutes. Each tube was then vortexed for 15 to 30 seconds, and the samples were centrifuged at 14000 x g for 10 minutes at 4°C. The supernatant was collected in a siliconized microfuge tube and stored at -80°C until use.

Free glycerol assay

The principal of this assay is that during triglyceride catabolism, the liberated glycerol is released from the cell; thus, measuring released glycerol is an indirect measure of lipid catabolism. The aliquot of conditioned media taken above was used in the free glycerol assay. The standards were prepared from the glycerol standard solution (Sigma, St. Louis, MO, cat# G7793; 0.26 mg glycerol/ml) and ranged in concentration from 0.26 to 0.0325 mg/ml. In the assay, 200 μ l of the conditioned media was added to 800 μ l of free glycerol reagent (Sigma, St. Louis, MO, cat# F6428) in a 1.5 mL disposable cuvette. The blank for the procedure was free glycerol reagent alone. The free glycerol reagent reacts with the glycerol to produce a color and absorbance is read at 540 nm, as detected by the spectrophotometer (Shimadzu, Kyoto, Tokyo, model# UV-2501PC). The samples were compared to the untreated group and normalized to total cell count for each treatment, as determined by the proliferation assay.

CD36 siRNA transfection

Thermo Scientific DharmaFECT siRNAs were used to test if CD36 expression could be inhibited in the PCa cell lines. siRNA mix was prepared in RNAase free water. All the procedures were executed per manufacturer's protocol. Briefly, cells were plated at 15,000 cells/cm² in growth media (DMEM, 10% FBS, 1% P/S) and incubated overnight. siRNA was premixed with basal media, without P/S. Lipofectamine 2000, the transfection reagent (Invitrogen, cat # 11668), was also premixed in basal media without P/S. Both mixes were incubated at room temperature for 5 minutes. The two reagent mixes were then combined and incubated for 20 minutes. The growth media on the cells was aspirated and replaced with fresh growth media without P/S, as P/S interferes with the transfection reagent. The transfection was performed by adding the siRNA-Lipofectamine mixture to cells and incubating them at 37°C, 5% CO₂ for 24 h. The transfection media was then aspirated, and the cells were washed with PBS and basal media with P/S was added to cells. The cells were incubated at 37°C for 48 h. After the incubation period, conditioned media and cell lysate were collected, and cell proliferation was analyzed. CD36 levels were analyzed by Western blot and PEDF levels were assessed by enzyme linked immunosorbent assay (ELISA) as described below.

Protein quantification

Total protein levels in conditioned media and cell lysate samples were quantified by the Coomassie assay using the Coomassie reagent and prediluted BSA protein standards from Thermo-Pierce (Rockford, IL, cat# 1856209 and #23208, respectively). The standards ranged in concentrations from 0 µg/ml to 1500 µg/ml. Samples were prepared using 10 µl of conditioned media or a 1:2 dilution of cell lysate in PBS. If the sample was too dilute, or too concentrated, the dilution was

adjusted. The samples were incubated for 5 minutes. Each sample tube was then compared to the high and low standard tubes to check if the sample was in range of the standard curve. If not, a new dilution was prepared accordingly to ensure the sample was in range of the standards. Standards or samples (200 μ L) were pipetted into the wells of a 96 well plate, with each sample in duplicate wells. The plate was read at 595 nm using a plate reader (BioTek, Synergy HT). Sample concentrations were calculated from the standard curve. This quantification function was incorporated into the plate reader assay.

PEDF levels

PEDF protein levels in conditioned media and cell lysate were quantified by Enzyme-linked immunosorbent assay (ELISA; BioProducts MD, Middletown, MD, cat# PED613) per the manufacturer's instructions. Briefly, all standards were prepared by serial dilution and were pipetted in duplicate wells on the microtiter plate (100 μ l / well). After all samples (100 μ l / well) and standards were added, the plate was incubated for 1 h at 37°C. At the end of this incubation, the samples were aspirated and each well was washed with 1X wash buffer five times using a squirt bottle, with the final wash aspirated. PEDF detector antibody (100 μ l / well) was then added to each well except the blank wells. This was incubated for 1 h at 37°C. At the end of incubation period, the detector antibody was aspirated and each well was washed as above. Next, 100 μ l / well of streptavidin peroxidase working solution was added. The plate was then incubated for 30 minutes at 37°C. Post this, wells were aspirated of their constituents and washed as above. Pre-warmed 3,3',5,5'-tetramethylbenzidine (TMB) substrate (100 μ l / well) was added to all wells, and this was incubated for 20 minutes at room temperature. Stop solution was then added (100 μ l / well) and a color change was observed from blue to yellow. The plate was

read at 450 nm using a microplate reader (BioTek, Synergy HT), and well concentrations were determined by reference to the standard curve. These calculations were incorporated into the plate reader assay. Samples were compared to the negative control, and when multiple assays were combined, samples were compared as fold over values.

Analysis of CD36, fyn, JNK and p38 protein levels by Western blot

Forty μg of each protein sample was prepared with 1X Laemmli sample buffer (BioRad, Hercules, CA, cat# 161-0737) in a 30 μl volume. Samples were then incubated at 95°C to ensure denaturation and then each sample loaded on a 12% precast acrylamide gel (Bio-Rad, Hercules, CA, cat# 456-9033). Prestained protein marker (10 μl) was also loaded as a size standard (Bio-Rad, Hercules, CA, cat# 161-0374). Gels were run at 120 volts for 1.5 h in Tris-glycine-SDS buffer (Bio-Rad, Hercules, CA, cat# 161-0732). The gel was then washed with transfer buffer (1X Tris-glycine and 20% methanol) for 10 minutes. The proteins separated on the gel were transferred to a solid PVDF membrane (GenHunter Corporation, Nashville, TN, cat# B301-50) by electroblotting for 2 h at 50 volts. To block non-specific binding, the membrane was then incubated with 1X Tris buffered saline (TBS, Sigma, St. Louis, MO, cat# 021M6078) with 0.05% tween (Sigma, St. Louis, MO, cat# P2287), TBS-T, and 5% dry milk at room temperature for 1 h. Post blocking, membranes were washed twice for 10 minutes each with 50 ml of TBS-T. The membrane was then probed with primary antibodies purchased from Cell Signaling (anti-CD36, cat# NBP1-8392, anti-fyn, cat# 4023, anti-p38, cat# 9212 or anti-JNK, cat# 3708). Primary antibodies were incubated in TBS and 5% bovine serum albumin (BSA) overnight at 4°C on a shaker. The next day, membranes were washed thrice for 10 minutes each with 50 ml TBS-T. Membranes were then incubated with secondary

antibody conjugated to horseradish peroxidase (HRP) at 1:1000 in TBS-T (ThermoScientific, Waltham, MA, cat# 34080) for 1 h at room temperature. At the end of 1h, membranes were washed thrice for 10 minutes each with 50 ml TBS-T. For chemiluminescence detection, the Pierce Pico reagents (ThermoScientific, Rockford, IL, cat# 34080) were used per manufacturer's instructions. Blots were then subjected to autoradiography using X-ray film for a permanent record.

Microvascular endothelial cell proliferation assay

Microvascular endothelial cells were plated in endothelial cell growth media at 5,000 cells/well in 100 μ l media in a 96 well gelatinized culture plate in growth media (day-1). Cells were incubated for 12-24 h to allow attachment. At day 0, media was aspirated and replaced with endothelial basal media (EBM Lonza, cat#: CC-3156) with 1% P/S with or without conditioned media samples in quadruplicate wells. For the negative proliferation control, basal media (serum and growth factor free) was used. For the positive control, growth media (EGM) was used. On day 7, proliferation was analyzed using the 3-(4,5-Dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent. Cells were aspirated of their media and 100 μ l of fresh serum-free media was added. Post this, 10 μ l of MTT stock solution (Sigma, St. Louis, MA, cat# M5655) was added to each well. The plates were then incubated at 37°C for 4 h. Following incubation, 85 μ l of solution was aspirated and 50 μ l of dimethyl sulfoxide was added to each well. This solution was mixed well by pipetting up and down. The plates were then incubated at 37°C for 10 minutes. The absorbance of each plate was read on the microplate reader at 540 nm (BioTek, Synergy HT). Samples were compared to the negative control for analysis.

Mouse prostate tissue analysis

The mouse prostate is comprised of 3 lobes called the dorsal-lateral, ventral and anterior prostate. All three lobes were collected from both wildtype C57BL/6 mice and TSP-1 knockout (KO) mice. Mice were anesthetized using isoflurane followed by a cervical dislocation to ensure death. A midline incision down the length of the abdomen was then performed to expose the internal organs. The intestines were moved to the side that exposed the reproductive system. Under a dissection microscope, the prostate lobes were excised, placed in microfuge tubes and kept on ice. Tissue samples were weighed and Tissue Extraction Reagent I (Invitrogen, Grand Island, NY, cat # FNN0071) was added (100 μ l per lobe) to the tissue. Tissue was homogenized using a hand-held Kontes pellet pestle motor and disposable pestles (Fischer Scientific, Waltham, MA, cat# KT 749540 and cat# KT-749521-1590, respectively) for 1 minute on ice. The sample was then centrifuged at 10,000 rpm for 5 minutes at 4°C to pellet cellular debris. The supernatant was collected and stored in a siliconized tube at -80°C until analysis. PEDF levels were quantified by ELISA as described above.

Statistical analysis

Statistical significance of the data was determined using ANOVA and Student t- tests with P values < 0.05 considered significant. Analysis was computed using the Systat statistical software incorporated into SigmaPlot program (version 12.0).

RESULTS

Specific aim 1: To establish that TSP-1 induces PEDF expression through inhibition of CD36 by (a) Determining if PEDF expression increases in PCa cells in presence of TSP-1 or anti-CD36 antibody treatment; (b) Assessing if PEDF levels increase in the presence of anti-CD36 siRNA treatment; and, (c) Comparing the levels of PEDF expression in prostate tissues taken from TSP-1 and CD36 knockout mice to that of wildtype mice.

A. Results for specific aim 1a

For TSP-1 and anti-CD36 antibody treatment studies, cells were plated on day 1, and treated with either TSP-1 at a dose of 1, 5, 10 or 20 nM or neutralizing anti-CD36 antibody at a dose of 2 or 5 µg/ml. Basal media alone served as a negative control. At the end of the 48 h treatment, cells were collected for proliferation analysis and cell lysate and conditioned media were collected. All cell culture experiments were repeated at least two times.

Part 1. TSP-1 Treatment

The effect of TSP-1 treatment on cell proliferation and viability

Limitless replicative potential is a key hallmark of cancer [59]. Proliferative activity is constantly kept in check in normal cells by endogenous inhibitors and cell cycle regulators, as reviewed in [59]. Loss of TSP-1 expression has been associated with a proliferation advantage in PCa cells [29]. TSP-1 expression mediates anti-angiogenic activity in normal and cancerous prostatic epithelia [46, 47, 83]. To determine if TSP-1 treatment affected cell proliferation of the normal prostate epithelial cell line (RWPE-1) or of PCa cell lines (LNCaP, PC-3 and DU145) direct cell counts were performed, with a trypan blue exclusion assay, on a Cellometer. In

RWPE-1 cells, TSP-1 treatment did not significantly change total cell number when compared to untreated cells (Figure 3A). Similarly, there was not a significant change in the viability of these cells upon treatment with TSP-1 (Figure 3B). Interestingly, LNCaP cells showed a significant increase in total cell count with TSP-1 treatment at all doses as compared to untreated (Figure 4B; $P \leq 0.05$), while there was no change in cell number with TSP-1 treatment compared to untreated (Figure 4B).

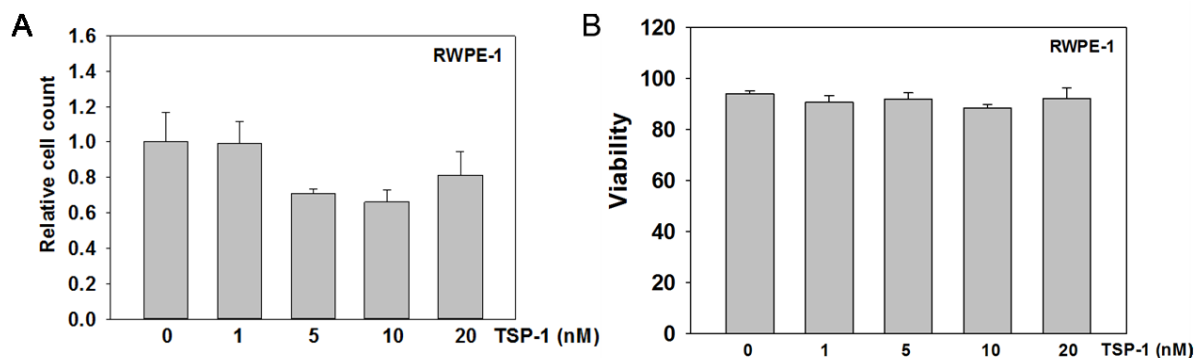


Figure 3. The effect of TSP-1 treatment on proliferation and viability of RWPE-1 cells. Total cell number and viability were determined in RWPE-1 cells after TSP-1 treatment. **(A)** Total cell number was measured by direct cell counts on a Cellometer and data presented as fold over untreated. **(B)** Viability was determined on the Cellometer with a trypan blue exclusion assay. The results presented in each graph are the combination of independent two experiments. No statistically significant differences were observed.

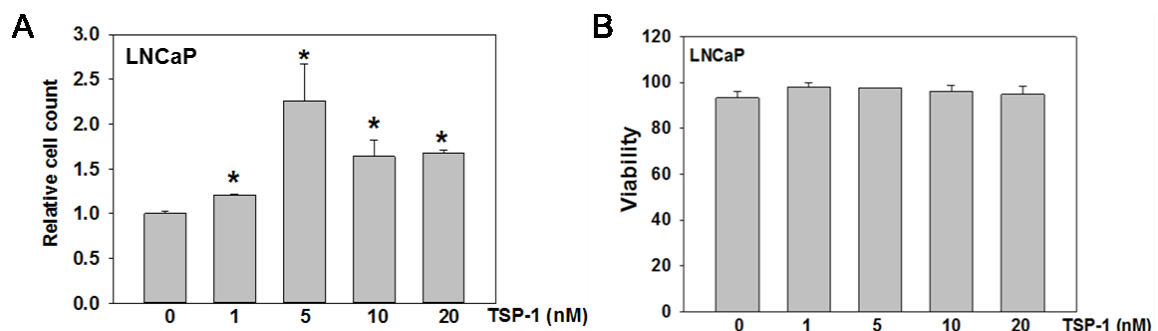


Figure 4. The effect of TSP-1 treatment on proliferation and viability of LNCaP cells. Total cell number and viability was determined in LNCaP cells after TSP-1 treatment. **(A)** Total cell number was measured by direct cell counts on a Cellometer and data presented as fold over untreated (*significantly increased compared to untreated, $P \leq 0.05$, **(B)** Viability was determined by trypan blue exclusion assay on the Cellometer, and there was no difference between treatment groups. Each experiment was performed at least 2 times with similar results.

When the more aggressive cell line, PC-3, was analyzed for cell proliferation, a biphasic response to TSP-1 treatment was observed (Figure 5A; $P \leq 0.041$). There was an increase in proliferation at the TSP-1 5 nM dose accompanied by a decrease at the 10 nM dose. However, the viability of PC-3 cells was unaffected by the TSP-1 treatment (Figure 5B). In contrast to the PC-3 cells, proliferation in DU145 cells was significantly decreased at the 1, 10 and 20 nM doses as compared to untreated ($P \leq 0.031$; Figure 6A). The viability in DU145 cells with TSP-1 treatment showed a modest dose-responsive increase (ANOVA, $P = 0.014$; Figure 6B). These results demonstrate that the effects of TSP-1 are variable, depending on cell line. With the normal cell line, RWPE-1, no effect was seen on neither cell proliferation nor viability. An increase in LNCaP cell number, with no change in cell viability, suggests that TSP-1 increases the rate of mitosis.

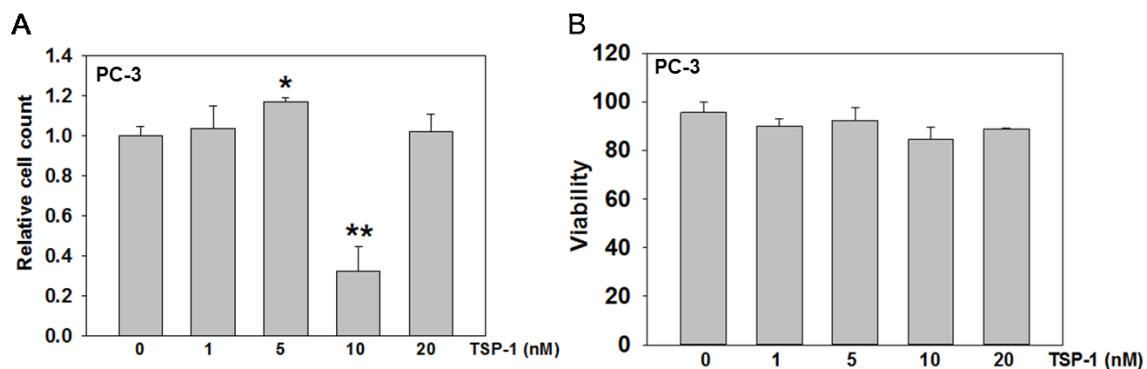


Figure 5. The effect of TSP-1 treatment on proliferation and viability in PC-3 cells. Total cell number and viability was determined in PC-3 cells after TSP-1 treatment. **(A)** Total cell number was measured by direct cell counts on a Cellometer and data presented as fold over untreated (*significantly increased compared to untreated, $P = 0.041$; **significantly decreased compared to untreated, $P = 0.018$). **(B)** Viability was determined on the Cellometer with trypan blue exclusion assay. No significant differences were observed between treatment groups. The experiment was performed at least 2 times with similar results.

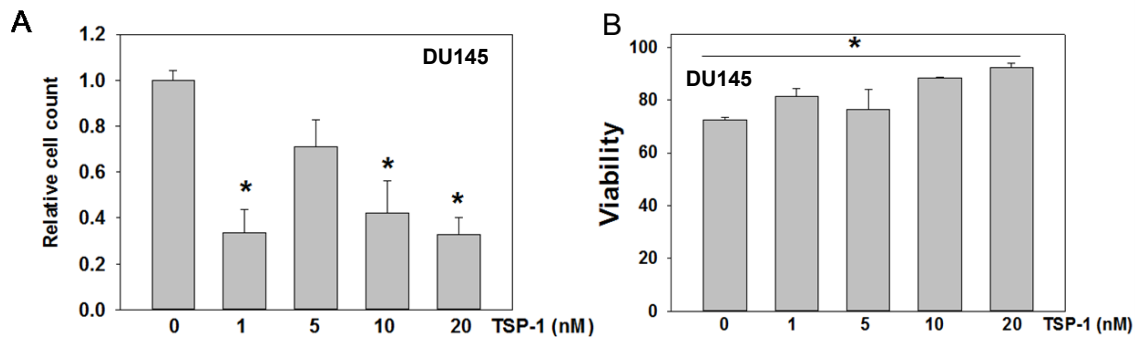


Figure 6. TSP-1 treatment decreased total cell count but increased viability in DU145 cells. Total cell number and viability was determined in DU145 cells after TSP-1 treatment. **(A)** Total cell number was measured by direct cell counts on a Cellometer and data presented as fold over untreated (* $P \leq 0.031$ compared to untreated). **(B)** Viability was determined on the Cellometer with trypan blue exclusion assay. Viability illustrated a dose response relationship between treatment groups (* $P = 0.014$ by ANOVA).

The effect of TSP-1 treatment on PEDF levels

Previous studies have shown that PEDF regulates lipid metabolism in PCa cells (Doll *et al*, unpublished observation). Based on my hypothesis, TSP-1 binding to the CD36 receptor would increase PEDF levels. To test this hypothesis, PEDF levels were quantified by ELISA in both the serum-free conditioned media and cell lysates of TSP-1 treated cells.

In RWPE-1 cells, repeat experiments produced somewhat differing results for PEDF expression. In the first experiment, an increase in PEDF expression was seen with 1, 5 and 20 nM TSP-1 doses, but not in the 10 nM dose ($P \leq 0.05$; Figure 7A). In the repeat experiment, there was a modest decrease in PEDF expression observed at the 1 nM dose; however, an increase in PEDF levels were observed at the 10 nM dose as compared to untreated ($P \leq 0.05$; Figure 7B). Although PEDF levels between experiments varied, it seems that the overall effect of TSP-1 was to increase secreted PEDF in these cells. In contrast, TSP-1 appeared to decrease PEDF in the cell lysates at 5-20 nM TSP-1 (Figure 7C; $P \leq 0.05$), although this effect was not observed in a repeat experiment (Figure 7D).

In LNCaP PCa cells, TSP-1 treatment showed a steady increase in PEDF levels in the serum-free conditioned media; however, this only reached significance at the 5 and 20 nM doses as compared to untreated cells ($P \leq 0.037$; Figure 8A). In the cell lysates, low dose TSP-1 (1 nM) decreased PEDF levels; however, 20 nM TSP-1 increased PEDF levels ($P \leq 0.043$; Figure 8B). Similarly to the results obtained for LNCaP cells, TSP-1 treatment increased PEDF in both the serum free conditioned media and in the cell lysates of PC-3 cells, although only the cell lysate data reached statistical significance (ANOVA, $P=0.009$; Fig 8C,D). In DU145 cells, a dose response increase in PEDF expression was seen in the serum free conditioned media with TSP-1 treatment (ANOVA, $P=0.004$; Figure 8E). However, with low dose TSP-1 (1nM) intracellular PEDF expression was decreased compared to untreated cells, but at higher doses, it was not significantly changed (1nM, $P \leq 0.015$; Figure 8F). These data show that, overall, TSP-1 treatment increased PEDF expression in all the PCa cell lines tested.

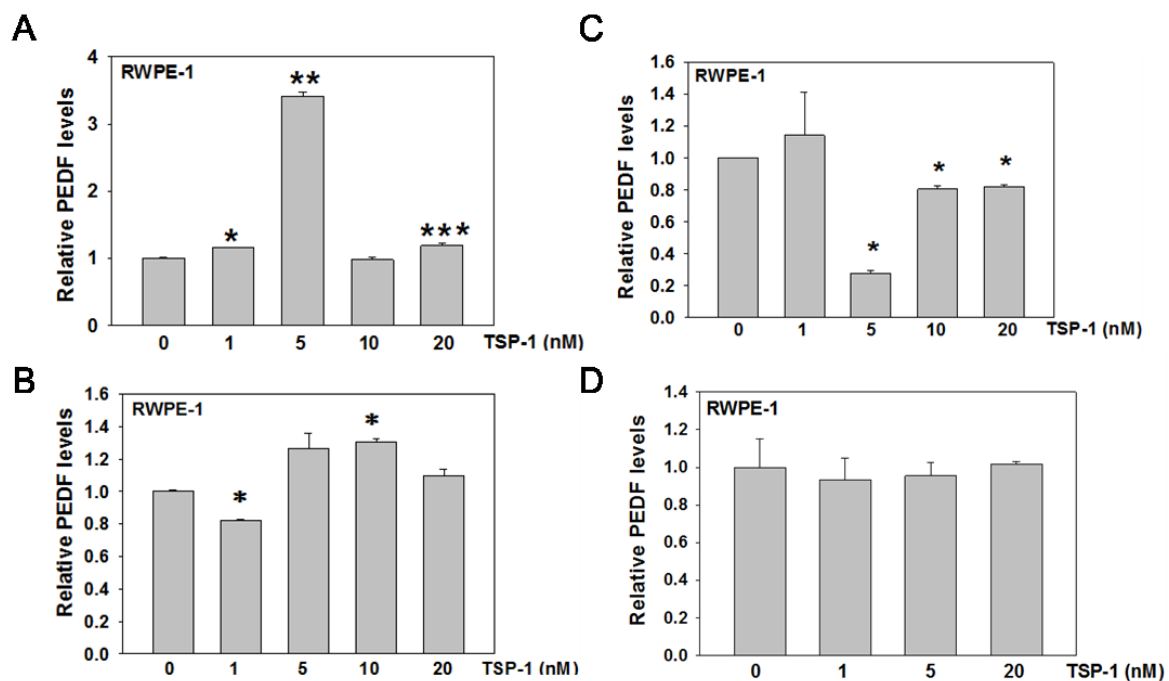


Figure 7. The effects of TSP-1 treatment on secreted and intracellular PEDF levels in RWPE-1 cells. After TSP-1 treatment, serum free conditioned media (A & B) and cell lysate (C & D) were collected and PEDF levels were measured by ELISA. The data are represented as fold over untreated (* $P \leq 0.05$, ** $P \leq 0.05$, *** $P \leq 0.05$ compared to the untreated sample). Results shown are two experimental repeats on this cell line, which show differing results.

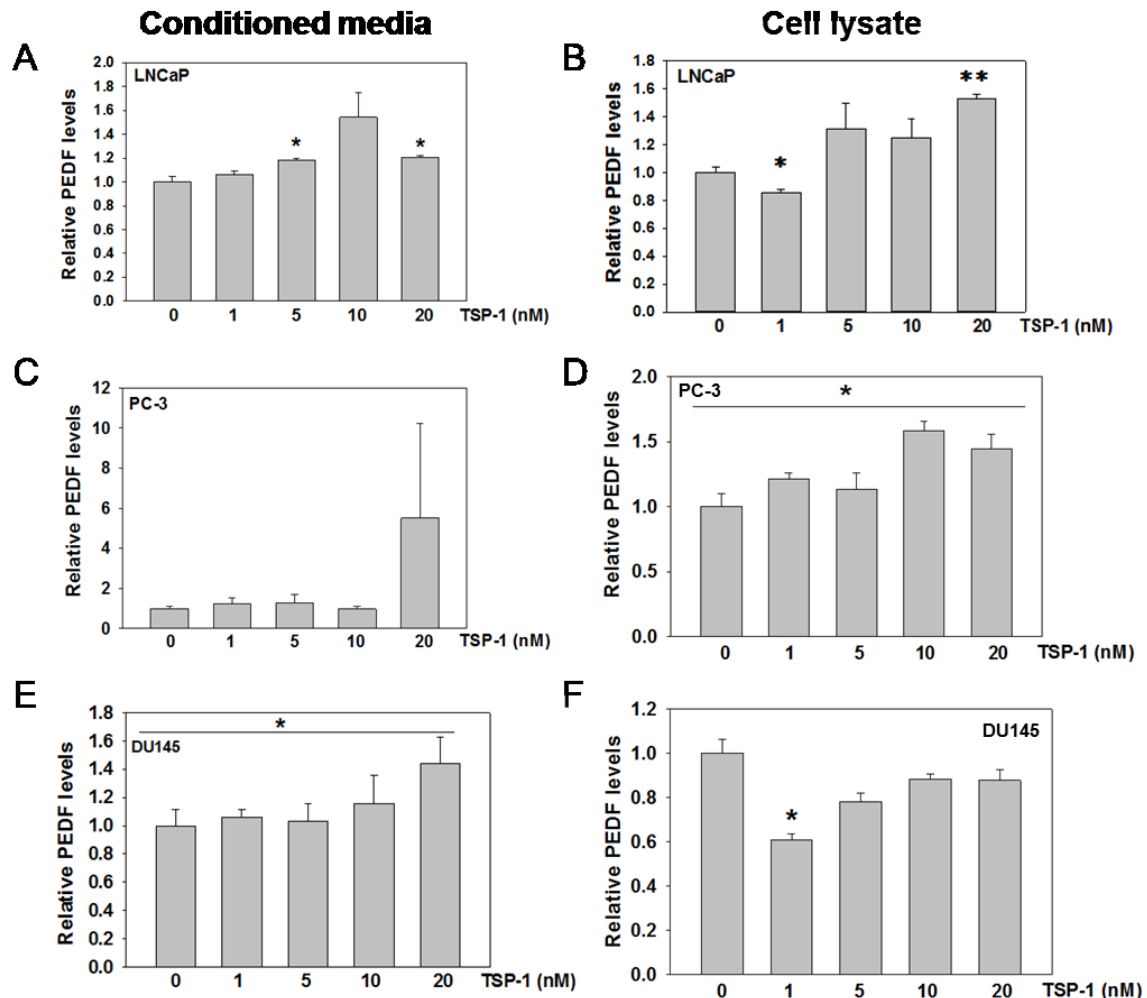


Figure 8. TSP-1 treatment effect on secreted and intracellular PEDF levels in PCa cells. After TSP-1 treatment, conditioned media and cell lysates were collected and PEDF levels were measured by ELISA. Data is represented as fold over untreated. **(A)** In LNCaP cells, TSP-1 increased PEDF expression in serum free conditioned media ($*P \leq 0.037$ compared to untreated). In the cell lysate **(B)** PEDF expression decreased at 1 nM and increased at 20 nM ($*P \leq 0.043$, $**P < 0.05$). **(C)** In the serum free conditioned media from PC-3 cells, no change in PEDF expression was observed however, in the cell lysate, a dose-response relationship with increasing PEDF was seen ($*P = 0.009$) **(D)**. In the serum free conditioned media from DU145 cells **(E)**, a dose-response increase was observed ($*P \leq 0.015$). In the cell lysate **(F)**, PEDF expression at was decreased 1 nM dose ($*P \leq 0.015$). Each experiment was done twice and similar results were obtained.

The effects of TSP-1 on lipolytic activity

The class B scavenger receptor CD36 recognizes a large variety of ligands , such as free fatty acids and oxLDL [80], and it functions in lipid uptake in endothelial cells [66, 80]. Moreover, CD36-null mice have an altered lipid profile [84]. However, as illustrated in Figure 2A and B, neither TSP-1 nor anti-CD36 antibody treatment altered lipid uptake in PC-3 and DU145 cells. According to my hypothesis, TSP-1

inhibitory binding to the CD36 receptor increases PEDF levels in prostate cells. Because PEDF has been previously shown to increase lipolysis in PCa cells (Doll *et al.*, unpublished observation), I measured lipolysis in TSP-1 treated cells using the free glycerol assay to quantify lipolytic activity. In RWPE-1 cells, TSP-1 increased lipolytic activity in a dose response manner (ANOVA, $P=0.001$; Figure 9A). Similarly, LNCaP cells also displayed a dose responsive increase with TSP-1 treatment (ANOVA $P\leq 0.011$; Figure 9B). In PC-3 cells, while there was a modest decrease in lipolytic activity at the lowest TSP-1 dose (1 nM; $P=0.037$), the 10 and 20 nM doses significantly increased lipolytic activity compared to untreated cells, with the highest activity at the 10 nM ($P\leq 0.015$; Figure 10A). In contrast to PC-3 cells, in DU145 cells, TSP-1 treatment decreased lipolytic activity significantly at the 1, 10 and 20 nM TSP-1 doses as compared to untreated cells ($P\leq 0.001$; Figure 10B). These data show that in normal prostate epithelial cells, as well as in the LNCaP and PC-3 PCa cell lines tested, TSP-1 stimulates lipid catabolism, but that in DU145 cells, the opposite effect occurs.

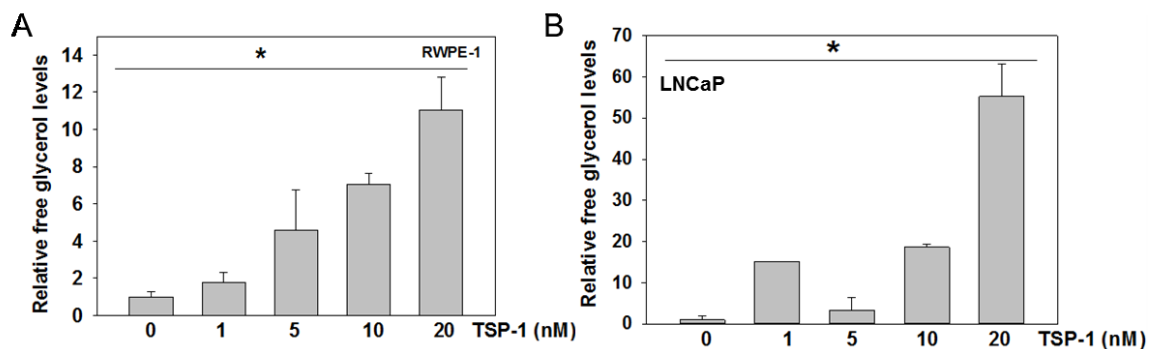


Figure 9. TSP-1 treatment increased lipolytic activity in RWPE-1 and LNCaP cells. Free glycerol levels were measured in serum free conditioned media collected after TSP-1 treatment in RWPE-1 (A) and LNCaP (B) cells. All measures were normalized to cell count and compared to untreated as fold over values ($*P\leq 0.011$ by ANOVA). Each experiment was performed twice with similar results.

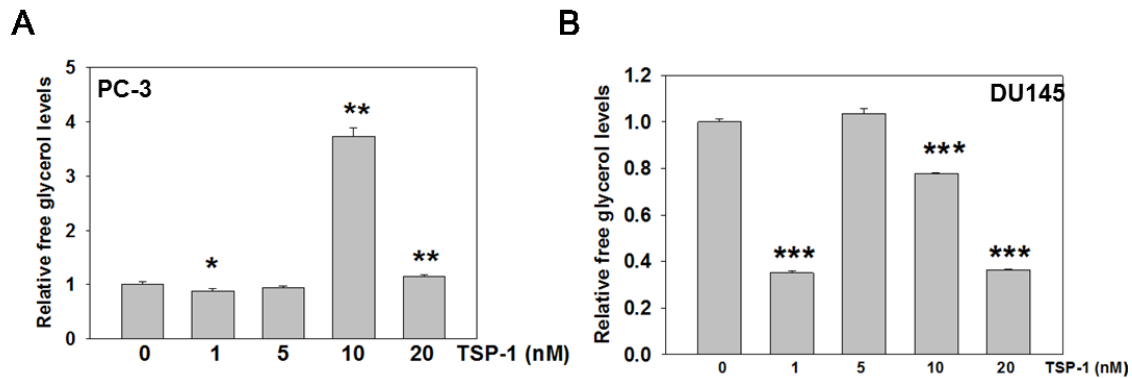


Figure 10. TSP-1 treatment had variable effects on lipolytic activity in PC-3 and DU145 cells. Free glycerol levels were measured in serum free conditioned media collected after TSP-1 treatment in PC-3 cells (A) and DU145 cells. (B) All measures were normalized to cell count and compared to untreated as fold over values (* $P=0.037$; ** $P<0.001$; *** $P<0.001$). Each experiment was performed twice with similar results.

Part 2. CD36 treatment

Effect of CD36 inhibition on cell proliferation

It has been previously shown that TSP-1 binding to CD36 blocks lipid uptake in endothelial cells [80] and promotes apoptosis [64]. CD36 is overexpressed in PCa cells [85], but its role in tumorigenesis remains unclear. To determine if blocking the CD36 receptor affected proliferation of normal prostate epithelial cells or of PCa cells, direct cell counts were performed, with a trypan blue exclusion assay, as described above for TSP-1 treatments. In RWPE-1 cells, blocking the CD36 receptor decreased cell proliferation; however, significance was only achieved with the 5 $\mu\text{g}/\text{ml}$ dose compared to untreated cells ($P=0.036$; Figure 11A). Similarly, cell viability was also decreased at all doses of the antibody compared to untreated ($P\leq 0.007$; Figure 11B). LNCaP cells presented with a similar pattern, with decreasing proliferation with neutralizing CD36 antibody treatment; however, the data did not achieve significance (Figure 12A). Viability in the LNCaP cells was decreased significantly in a dose-dependent manner by the antibody treatment (ANOVA, $P=0.001$; Figure 12B).

In PC-3 cells, blocking the CD36 receptor did not significantly change proliferation when compared to untreated (Figure 13A). Similarly, there was no

significant change in viability in these cells with CD36 receptor inhibition (Figure 13B). Similar to the PC-3 cells, in DU145 cells, treatment with blocking anti-CD36 antibody did not significantly change total cell number when compared to untreated cells (Figure 14A). Interestingly, however, viability in DU145 cells was decreased in a dose-dependent manner with CD36 receptor blockade (ANOVA, $P=0.037$; Figure 14B). Thus, in these cells, CD36 blockade may be increasing apoptosis or another cell death pathway.

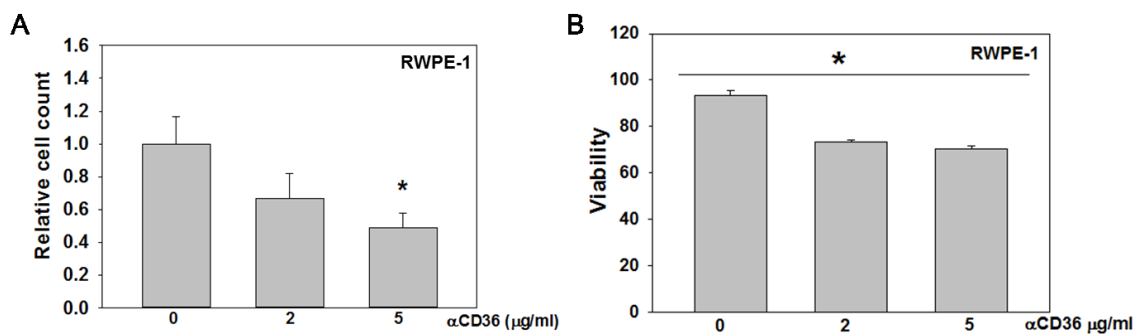


Figure 11. Blocking CD36 receptor decreased proliferation and viability in RWPE-1 cells. Total cell number and viability was determined in RWPE-1 cells after neutralizing anti-CD36 antibody treatment. **(A)** Total cell number was measured by direct cell counts on a Cellometer and data are presented as fold over untreated ($*P= 0.036$). **(B)** Viability was determined on the Cellometer with trypan blue exclusion assay ($*P= 0.001$ by ANOVA). Each experiment was done twice and similar results were obtained.

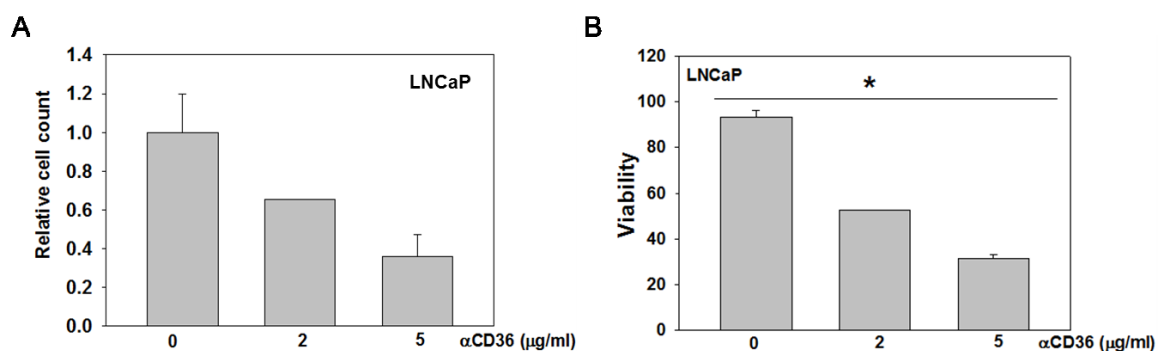


Figure 12. Blocking the CD36 receptor did not change proliferation but decreased the viability of LNCaP cells. Total cell number and viability was determined in LNCaP cells after neutralizing anti CD36 antibody treatment. **(A)** Total cell number was measured by direct cell counts on Cellometer and data presented as fold over untreated. Though a trend toward decrease was observed, results were not statistically significant ($P=0.057$ by ANOVA). **(B)** Viability was determined on the Cellometer with trypan blue exclusion assay. A dose responsive decrease in viability was observed with anti-CD36 antibody treatment ($*ANOVA, P= 0.001$). Each experiment was performed twice and similar results were obtained.

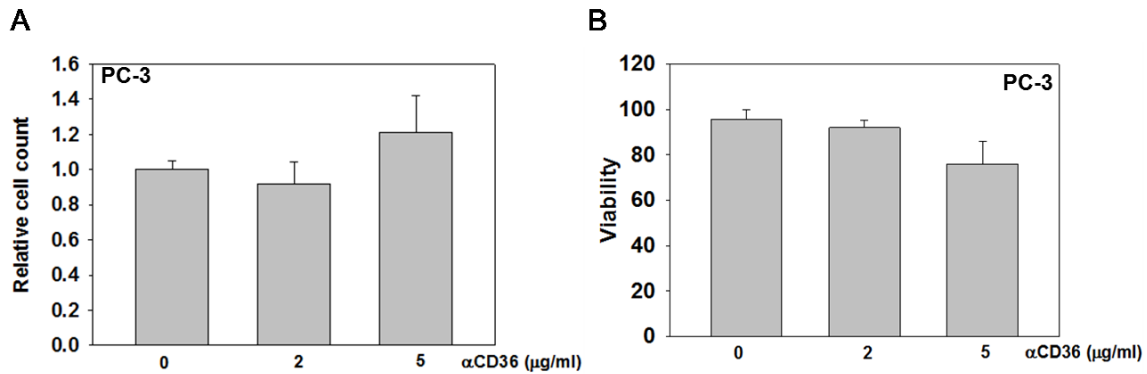


Figure 13. Blocking the CD36 receptor did not alter proliferation or viability of PC-3 cells. Total cell number and viability were determined in PC-3 cells after neutralizing anti-CD36 antibody treatment. **(A)** Total cell number was measured by direct cell counts on a Cellometer and data presented as fold over untreated. The slight increase at the 5 µg/ml dose was not statistically significant. **(B)** Viability was determined on the Cellometer with trypan blue exclusion assay. No significant differences were observed with treatment. Each experiment was done twice and similar results were obtained.

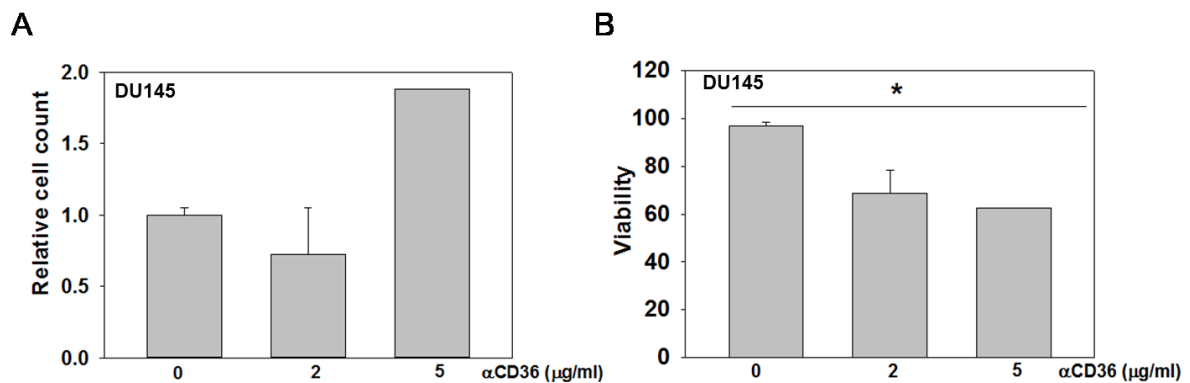


Figure 14. Blocking CD36 receptor results did not change proliferation but decreased viability of DU145 cells. Total cell number and viability were determined in DU145 cells after anti-CD36 antibody treatment. **(A)** Total cell number was measured by direct cell counts on a Cellometer and data presented as fold over untreated. **(B)** Viability was determined on the Cellometer with trypan blue exclusion assay (* $P = 0.037$ by ANOVA). Each experiment was done twice and similar results were obtained.

Effect of CD36 inhibition on PEDF levels

To establish if blocking the CD36 receptor in PCa cells increases PEDF expression, PEDF levels were quantified by ELISA in both the serum-free conditioned media and cell lysates collected from neutralizing anti-CD36 antibody treated cells. In the normal prostate epithelial cell line, RWPE-1, there was an increase in secreted PEDF levels at the 5 µg/ml dose compared to untreated cells ($P = 0.002$; Figure 15A).

In LNCaP PCa cells, blocking the CD36 receptor resulted in a modest increase in secreted PEDF at both doses of the antibody; however, significance was only achieved at the lower dose ($P=0.042$; Figure 15B). Similarly, PC-3 cells showed a significant increase in PEDF expression at both doses of neutralizing antibody in the serum free conditioned media ($P=0.007$; Figure 15C). Likewise, in DU145 cells, there was a significant dose-responsive increase in PEDF levels in the serum free conditioned media when cells were treated with anti-CD36 antibody (ANOVA $P\leq 0.001$; Figure 15D).

For several of the cell lines, the opposite results were observed in the cell lysate. In the RWPE-1 cell lysate, a high dose (5 $\mu\text{g/ml}$) of the antibody decreased PEDF expression ($P\leq 0.05$; Figure 15E). Interestingly, in the LNCaP cell lysate, a low dose of neutralizing anti-CD36 antibody (2 $\mu\text{g/ml}$) increased PEDF expression ($P=0.022$; Figure 15F), while the higher dose decreased it compared to untreated cells ($P=0.005$; Figure 15F). In PC-3 cell lysate, PEDF expression was increased at both doses of neutralizing anti-CD36 antibody ($P\leq 0.001$; Figure 15G). In the DU145 cell lysate, PEDF expression was decreased at the low dose (2 $\mu\text{g/ml}$) as well as the high dose; however, significance was only achieved with the low dose ($P\leq 0.022$; Figure 15H).

These data suggest that, overall, blocking the CD36 receptor stimulates secreted PEDF expression in all prostate cell lines tested. There was more variability in the levels of intracellular PEDF with CD36 treatment. While it was largely increased at low doses (2 $\mu\text{g/ml}$) across the cell lines, the higher dose decreased intracellular PEDF levels in RWPE-1 and LNCaP cells while increasing it in PC-3 cells.

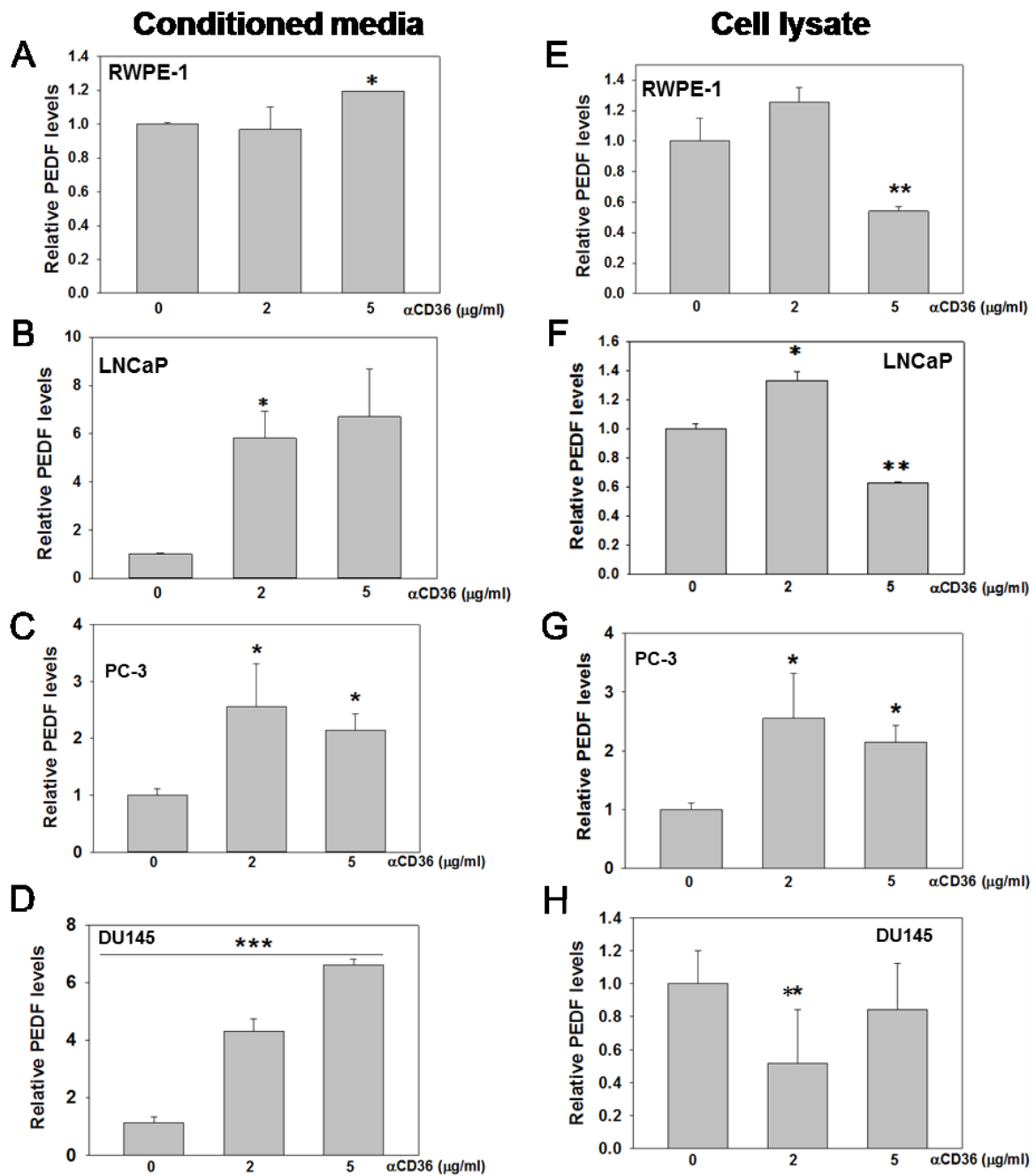


Figure 15. Blocking the CD36 receptor increases secreted PEDF levels. After neutralizing anti-CD36 antibody treatment, serum-free conditioned media and cell lysates were collected and PEDF levels were measured by ELISA. Data is represented as fold over untreated. Results for RWPE-1 (A,E), LNCaP (B,F), PC-3 (C,G) and DU145 (D, H) are shown for both conditioned media (A-D) and cell lysate (E-H) samples. PEDF levels increased in serum free conditioned media and some cell lysates compared to untreated (* $P \leq 0.022$). In DU145 cells, serum free conditioned media (D), the increase was dose responsive (**ANOVA $P \leq 0.001$). However, PEDF levels were decreased compared to untreated, $P \leq 0.051$.

Effect of CD36 inhibition on lipolytic activity

Because CD36 regulates PEDF levels and PEDF is known to increase lipolysis in PCa cells (Doll *et al.* unpublished data), I evaluated lipolytic activity in PCa cells upon blocking the CD36 receptor. I used the free glycerol reagent to quantify this activity for the TSP-1 treatments.

In RWPE-1 cells, there was a dose-responsive increase in lipolytic activity with neutralizing anti-CD36 antibody treatment (ANOVA $P \leq 0.001$; Figure 16A). Similarly, LNCaP cells also displayed an increase in lipolytic activity with increasing neutralizing anti-CD36 antibody doses; however, the data did not achieve significance (Figure 16B). Likewise, in PC-3 cells, lipolytic activity was increased with both doses of neutralizing anti-CD36 antibody ($P \leq 0.001$; Figure 17A). In contrast, when the CD36 receptor was blocked in DU145 cells, a significant decrease in lipolysis was seen at the 5 $\mu\text{g/ml}$ dose ($P \leq 0.001$; Figure 17B).

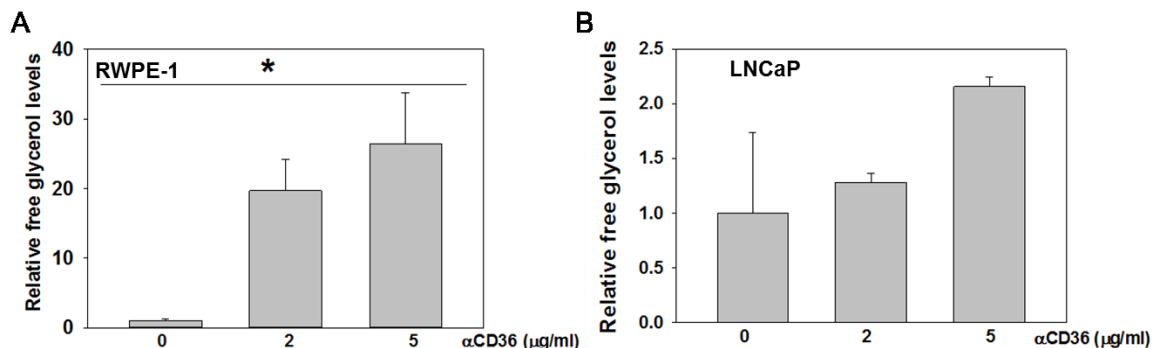


Figure 16. Blocking the CD36 receptor increased lipolytic activity in RWPE-1 cells and LNCaP cells. Free glycerol levels were measured in serum free conditioned media after neutralizing anti-CD36 antibody treatment in RWPE-1 (A) and LNCaP cells (B). All measures were normalized to cell count and compared to untreated as fold over values. In (A), lipolytic activity was significantly increased in a dose responsive manner ($*P \leq 0.010$ by ANOVA). The increase observed in LNCaP cells (B), was not statistically significant. Each experiment was performed twice with similar results.

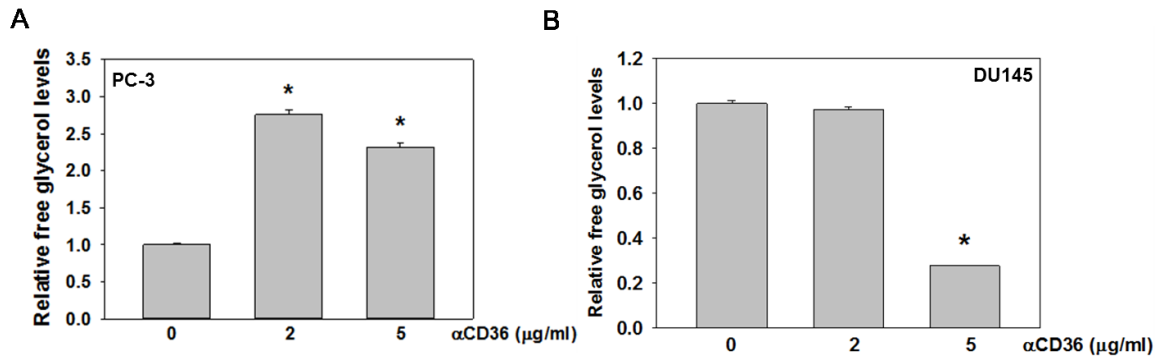


Figure 17. Blocking the CD36 receptor increased lipolytic activity in PC-3 cells but decreased it in DU145 cells. Free glycerol levels were measured in serum free conditioned media after neutralizing anti-CD36 antibody treatment in PC-3 (A) and DU145 cells (B). All measures were normalized to cell count and compared to untreated as fold over values. In PC-3 cells (A), lipolytic activity was significantly increased at both doses of antibody (* $P \leq 0.001$). In DU145 cells (B), lipolytic activity was significantly decreased at the 5 µg/ml dose, (* $P \leq 0.001$). Each experiment was performed twice with similar results.

B. Results for Specific aim 1b:

This aim was pursued to investigate if PEDF expression increased in PCa cells when CD36 receptor expression was blocked using an siRNA approach. I tested four different siRNAs against CD36 for this assay. DU145 cells were used to test this assay because blocking the CD36 receptor in these cells resulted in an increased secreted PEDF expression. Cells were plated overnight, transfected with siRNA on day 2 and then incubated for 2 days in basal media. On day 5, cell lysates and serum free conditioned media were collected to assay for CD36 and PEDF levels as well as cell proliferation and viability. GAPDH siRNA was used as a negative control for siRNA activity in this assay.

To confirm CD36 suppression, I performed a Western blot using anti-CD36 antibody on the cell lysates. Two different anti-CD36 antibodies were tested; however, due to technical difficulties with the assay and antibody, I was not able to confirm CD36 suppression (data not shown). Despite this, I still assessed cell proliferation and PEDF levels in these samples. Although several of the CD36 siRNAs suppressed total cell numbers ($P \leq 0.013$; Figure 18A; clone 7 at 10 nM; clone 8 at 10 nM and clone 9 at both 5 and 10 nM), cell numbers were also decreased with

the GAPDH siRNA at 5 nM as compared to untreated ($P=0.026$; Figure 18A), making any conclusions difficult. Conversely, none of the siRNAs affected cell viability (Figure 18B).

A PEDF ELISA was performed on the CD36 siRNA treated serum-free conditioned media and cell lysate samples. While siRNA against CD36 receptor diminished PEDF levels in serum-free conditioned media ($P\leq 0.023$; Figure 19A; clones 7,8 and 9 at both 5 and 10 nM; and clone 10 at 5 nM), the non-targeting siRNA used (GAPDH) also decreased secreted PEDF, so the significance of this data cannot be determined. Interestingly, the PEDF expression in the cell lysate was slightly increased with clone 8 at the 10 nM dose ($P= 0.047$; Figure 19B). Overall, an accurate interpretation of these results will not be possible until the CD36 levels can be assessed in these samples; however, the data with the GAPDH siRNA suggests that the assay needs to be further optimized to decrease non-specific effects.

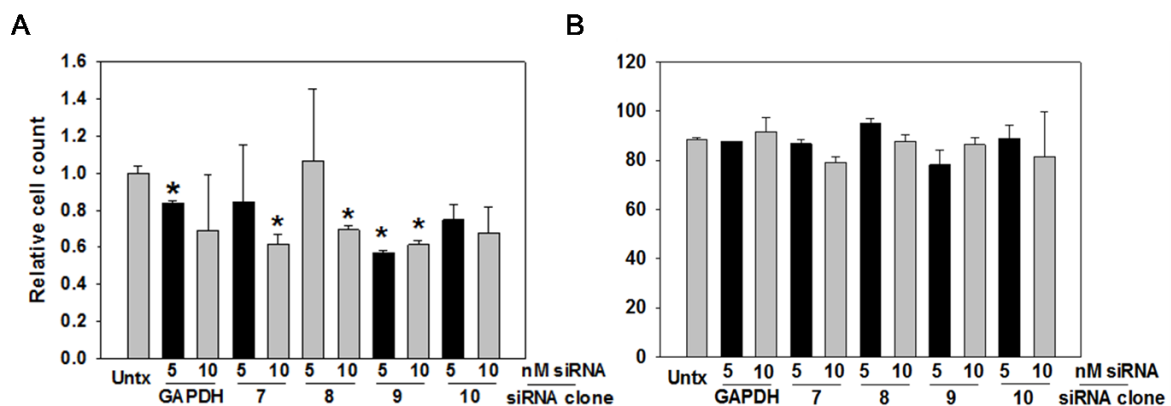


Figure 18. siRNA against CD36 receptor decreased proliferation in DU145 cells. Cells were plated overnight and then transfected with anti-CD36 siRNA for 24h. After additional 48h incubation, total cell number and viability were determined after anti-CD36 siRNA treatment. **(A)** Total cell number was measured by direct cell counts on a Cellometer. All measures were compared to untreated ($*P\leq 0.026$). **(B)** Viability was determined on the Cellometer with trypan blue exclusion assay. There was no significant difference between samples. Note: this experiment was performed only one time.

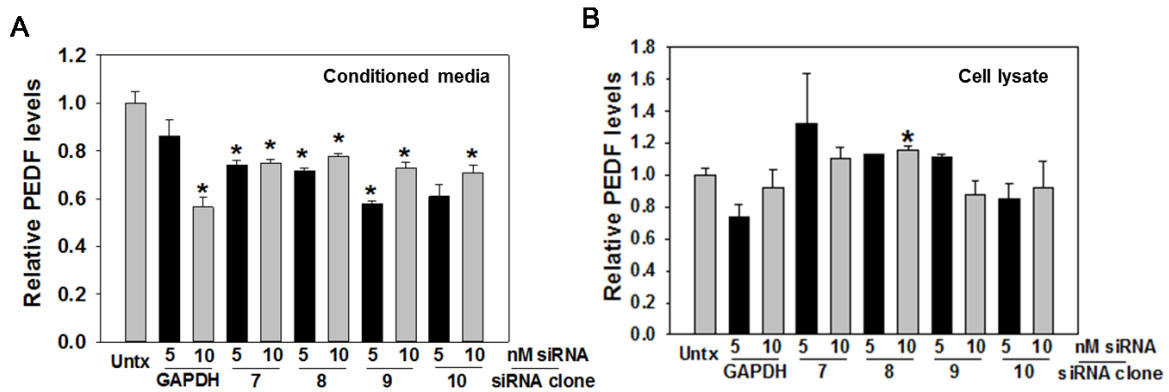


Figure 19. The effect of anti-CD36 siRNA on PEDF levels in DU145 cells. Cells were plated and transfected with anti-CD36 siRNA. PEDF ELISA was performed on the serum free conditioned media and cell lysate samples collected. **(A)** There was a decrease in secreted PEDF protein with all anti-CD36 clones, when compared to untreated ($*P \leq 0.023$). In cell lysate **(B)**, one CD36 siRNA clone (clone 8) at the 10 nM (clone 8), $*P = 0.047$.

Results for specific aim 1c:

In order to analyze if the loss of TSP-1 expression altered PEDF protein levels *in vivo*, I proposed to evaluate PEDF levels in TSP-1 and CD36 KO mice. However, due to prohibitive costs, CD36 knockout mouse tissues were not examined. I collected age-matched TSP-1 KO and wildtype prostate tissues by microdissection. The ventral and dorsolateral prostate lobe tissues were initially frozen and stored. Tissues were homogenized and proteins were extracted and examined by PEDF ELISA. Tissues were examined from mice at both 4 and 6 months of age and data were normalized to total protein content.

In 4 month old mice, there was a small trend toward a decrease in PEDF expression in both the ventral prostate and dorsolateral prostate of the TSP-1 KO (n=3) compared to the wildtype mice (n=3); however, data were not statistically significant (Figure 20A). At 6 months of age, there was no significant change in the PEDF expression levels between the TSP-1 KO (n=4) and age matched wildtype mice (n=4) (Figure 20B). Due to the large variations within the samples and the small sample size of the experiment, future experiments are needed to increase the

number of tissues analyzed per group to confirm whether or not loss of TSP-1 affects PEDF levels in the prostate *in vivo*.

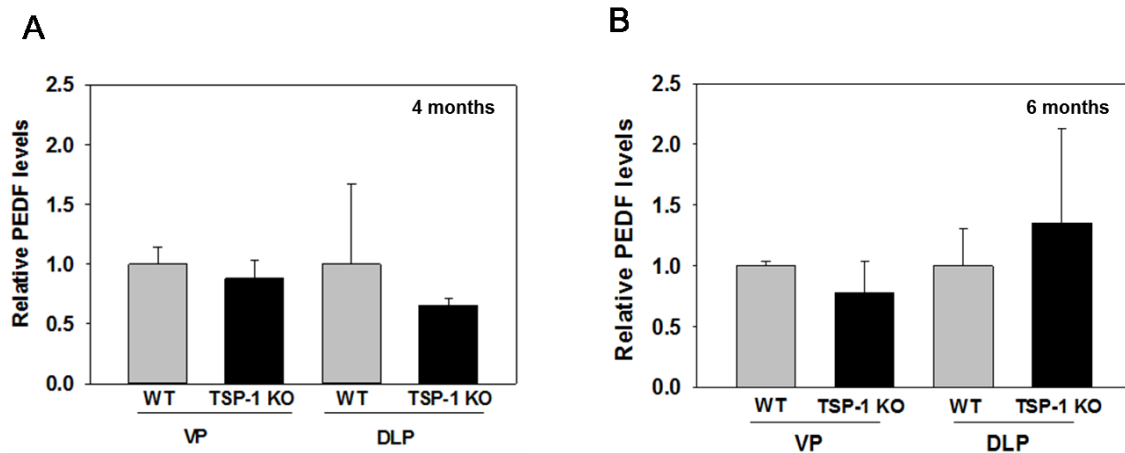


Figure 20. Loss of TSP-1 expression did not alter in PEDF expression in mouse prostate tissues. The ventral prostate (VP) and dorsolateral prostate (DLP) were collected from TSP-1 KO and wildtype mice, age matched at 4 months, n= 5 WT, 4 TSP-1 KO (A) and 6 months, n= 3 WT, 3 TSP-1 KO (B). PEDF levels were analyzed by ELISA. There was no statistically significant difference between the PEDF levels in the TSP-1 KO VP or DLP, as compared to wildtype VP and DLP.

Specific aim 2

To elucidate the signaling pathway through which TSP-1 and/or CD36 regulate PEDF expression and determine if the TSP-1-induced PEDF produces a more anti-angiogenic phenotype by (a) Examining the expression levels of known TSP-1 and CD36 signal mediators fyn, p38 and JNK; and, b) Evaluating changes in secreted angiogenic activity in PCa cells treated in aim 1 above.

Results for specific aim 2a

As depicted in Figure 1, the signaling molecules that mediate the TSP-1 - CD36 activity in endothelial cells are JNK, p38 MAPK and fyn [68]. This signaling pathway, however, has not been investigated in PCa cells. To investigate the potential role of signaling molecules in the TSP-1 - CD36 pathway, Western blot analysis was performed to detect the expression levels of these molecules in cell lysate samples derived from PC-3 and DU145 PCa cells treated with TSP-1 or anti-

CD36 antibody from Aim 1a. All protein levels were compared to GAPDH levels as a control for equal protein loading. No fyn was detected in untreated DU145 or PC-3 cells (Figure 21A,B). TSP-1 treatment did modestly increase fyn expression, particularly at the higher dose in both cell lines (Figure 21A,B). Neutralizing anti-CD36 antibody did not increase fyn levels in either cell line (Figure 21A,B).

My data shows that p38 was expressed in untreated PC-3 and DU145 cells (Figure 21A,B). However, TSP-1 treatment did not change p38 expression in either DU145 or PC-3 cells. In contrast, neutralizing anti-CD36 antibody decreased p38 expression at both doses in both DU145 and PC-3 cells (Figure 21A,B).

JNK was expressed in DU145 untreated cells (Figure 21A). However, in PC-3 cells, JNK expression was barely detectable in untreated cells (Figure 21B). TSP-1 treatment did not alter JNK expression in DU145 cells across all doses except at 5 nM, which showed decreased expression (Figure 21A). In PC-3 cells, TSP-1 treatment modestly increased JNK expression at 5 and 10 nM (Figure 21B). Neutralizing anti-CD36 antibody had no discernable effect on JNK expression at either dose in PC-3 cells (Figure 21B). However, in DU145 cells, JNK expression was diminished at 2 μ g/ml of neutralizing anti-CD36 antibody (Figure 21A). These data suggest that TSP-1 treatment induces expression of p38 MAPK, fyn and JNK in PCa cells. While, blocking CD36 receptor diminishes the expression of JNK and p38.

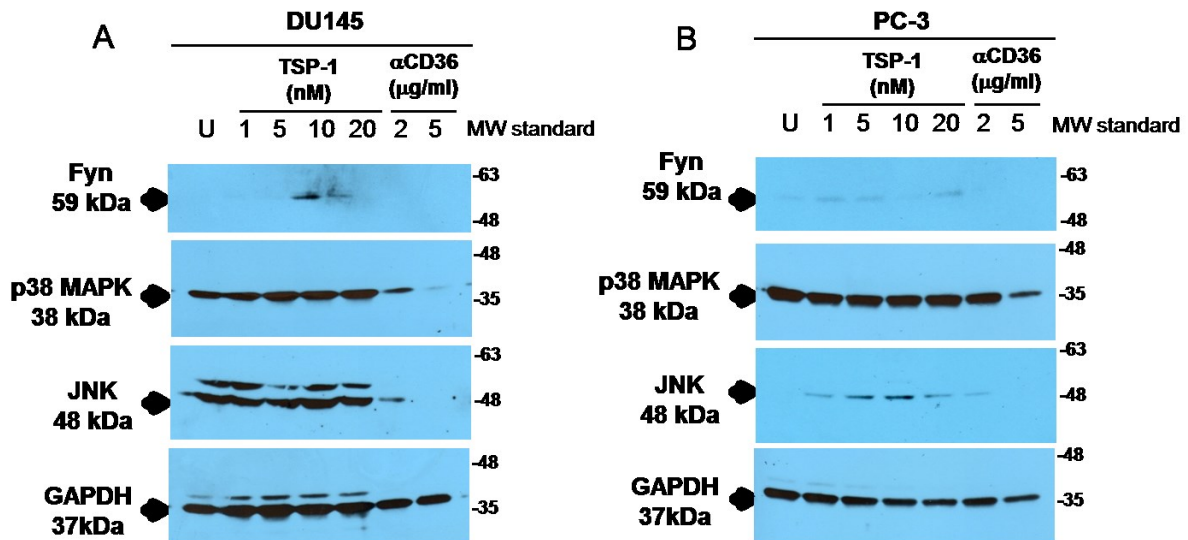


Figure 21. TSP-1 and anti-CD36 treatment alter intracellular signaling in prostate cancer cells. DU145 (A) and PC-3 (B) cells were treated with either TSP-1 or neutralizing anti-CD36 antibody in serum-free media for 48 h. Cell lysates were collected, quantified, and total proteins separated by SDS-PAGE (12% gel) samples were then electroblotted to a PVDF membrane and probed with the indicated antibody. GAPDH served as a control for protein loading. U, untreated; MW, Molecular weight markers (in kDa).

Results for specific aim 2b

To assess if TSP-1 treatment or blocking the CD36 receptor altered secreted angiogenic activity by prostate cells, I measured human microvascular endothelial cell (HMVEC) proliferation, by MTT assay. The samples tested were the serum-free conditioned media derived from LNCaP, RWPE-1, PC-3 and DU145 cells across different doses of TSP-1 and neutralizing anti-CD36 antibody collected in Aim 1a above. Endothelial cell growth media served as a positive control for this assay, while basal media served as the negative control. TSP-1 treatment did not alter the angiogenic activity, as measured by HMVEC proliferation, in any of the four cell lines (Figure 22A-D). Similarly, angiogenic activity was not altered in any of the four cell lines by neutralizing anti-CD36 antibody treatment (Figure 23A-D) However, these assays were only performed one time; thus, repeat experiments are needed to confirm the results obtained.

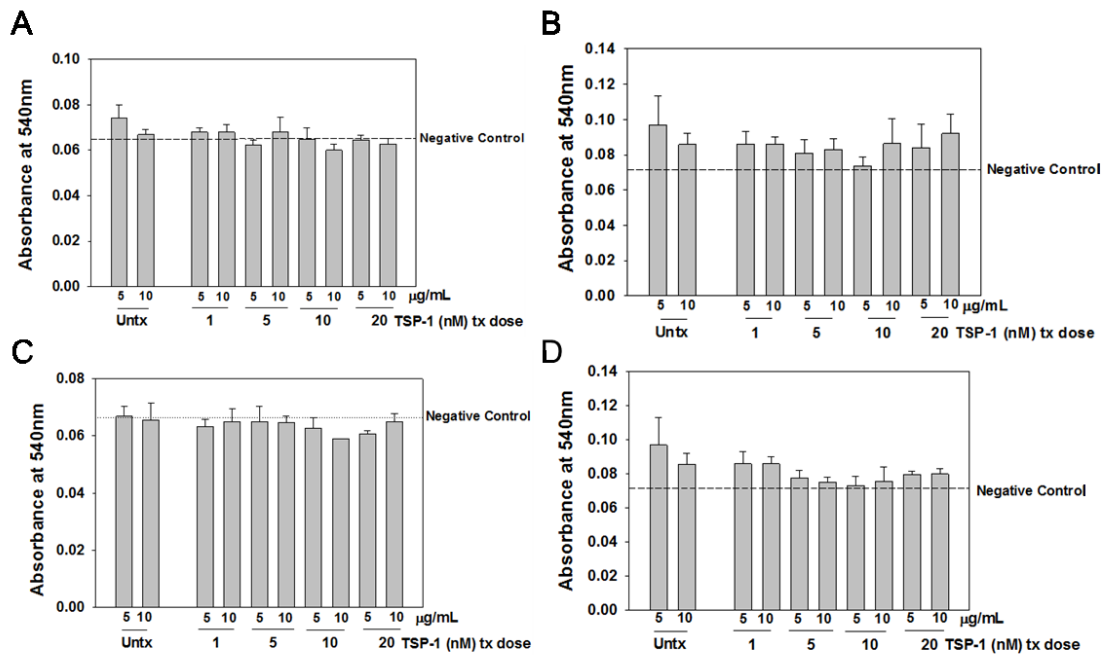


Figure 22. TSP-1 treatment did not alter secreted angiogenic activity of normal prostate epithelial or prostate cancer cells. Endothelial cells were plated in 96 well plates overnight and serum-free conditioned media (CM) derived from cells treated with TSP-1 was added to the wells. After 7 days, an MTT assay was performed and read on the plate reader. In RWPE-1 (**A**), PC-3 (**B**), LNCaP (**C**) and DU145 (**D**), there was no change in the endothelial cell proliferation. The negative control was basal media alone, and TSP-1 alone was tested alone as an additional control at 1, 5, 10 and 20 nM doses with no change on proliferation (data not shown).

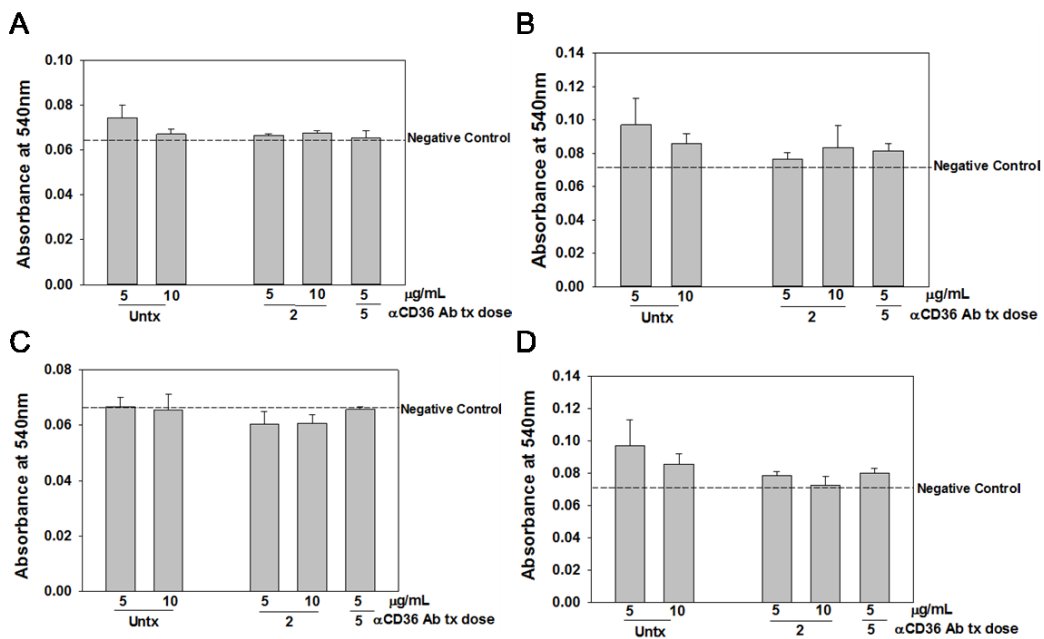


Figure 23. Anti-CD36 treatment did not alter secreted angiogenic activity of prostate cells. Endothelial cells were plated in 96 well plates overnight and serum-free conditioned media (CM) derived from PCa cells treated with alpha-CD36 antibody was added to the wells. After 7 days, MTT assay was performed and read on the plate reader. In RWPE-1 (**A**), PC-3 (**B**), LNCaP (**C**) and DU145 (**D**), there was no change in the endothelial cell proliferation with treatment. The negative control was basal media. Anti-CD36 antibody was tested at both the 2 and 5 µg/ml dose with no change on proliferation (data not shown).

DISCUSSION

The purpose of these studies was to determine if the TSP-1 - CD36 pathway regulates PEDF expression and angiogenic activity in prostate cells. TSP-1 and PEDF are both potent angiogenic inhibitors that are downregulated in PCa tissues [57, 86, 87]. In endothelial cells, TSP-1 mediates its anti-angiogenic activity via by binding to the CD36 receptor [88]. Studies in other tissues have established a regulatory loop between TSP-1 and PEDF. Guan *et al.* found that, when PEDF was overexpressed in glioma cells (U251), there was a 5.3 fold increase in TSP-1 expression [74]. Consistent with these results, Jia and Waxman found that KM12 colon tumor cells expressing PEDF displayed an increase in TSP-1 levels [75]. Also, in endometrial cancer cell lines, PEDF induced an increase in TSP-1 mRNA levels [76]. In a study by Aparicio *et al.* human umbilical vein endothelial cells exposed to cobalt chloride showed a reduction in TSP-1 transcripts and treatment with PEDF blocked this reduction [89]. Contrarily, Schmitz *et al.* show that PEDF overexpression in pancreatic cancer cells was associated with suppression of TSP-1 levels [72]. However, if TSP-1 influences PEDF expression in tissues is unknown, and no studies have illustrated the effects of TSP-1 treatment on PEDF expression on normal prostate epithelial cells or in PCa cells. In this study I hypothesized that TSP-1 - CD36 binding would stimulate PEDF expression.

In specific aim 1, I showed that TSP-1 treatment increased secreted PEDF protein expression in LNCaP and DU145 cells. In addition, the protein expression in PC-3 cells was somewhat increased but did not reach statistical significance. When treated with neutralizing anti-CD36 antibody, all of the PCa cells used in this study showed an increase in secreted PEDF expression. My data also suggested an overall increase in cellular PEDF expression levels in prostate cell lines at lower dose of the antibody, with the exception of DU145 cells.

PEDF has been most well studied as a secreted anti-angiogenic protein, and an increase in secreted PEDF may be linked to an increase in anti-angiogenic activity [39]. However, some studies have also detected intracellular protein levels and even nuclear staining [90-92]. Studies on retinoblastoma, neuroblastoma, hepatocarcinoma and retinal pigment epithelial cells have shown both cytoplasmic and nuclear PEDF expression levels [93]. Based on this, Anguissola *et al.* suggest that PEDF should be included in human serpins that demonstrate nucleocytoplasmic distribution such as antichymotrypsin and antiangiotensinogen [94]. Although a clear demarcation in function between secreted and intracellular PEDF expression is not well established, it is believed that the intracellular PEDF expression is linked to regulation of lipolytic activity in the cell [95]. My studies illustrate that LNCaP and RWPE-1 cells showed an increase in lipolytic activity with TSP-1 doses. DU145 cells showed a decreased lipolytic activity. Blocking the CD36 receptor in PC-3, LNCaP and RWPE-1 cells showed an increase in lipolytic activity. However, DU145 cells showed decreased lipolytic activity. CD36 signaling blocks lipolysis in most PCa cells and in normal prostate epithelial cells. However, the decreased lipolytic activity in DU145 cells suggests that a underlying difference in genetic background specific to this line alters the lipolytic signaling pathway.

Since tumor progression is guided by several factors such as cell proliferation, I determined whether treatment with TSP-1 or anti-CD36 antibody was altering cell proliferation and viability of prostate cells. My cell proliferation data showed that TSP-1 treatment did not alter the total cell number or viability in RWPE-1 cells. Data obtained from LNCaP cells revealed that there was an increase in total cell count with all the TSP-1 doses, although viability was unaffected. Thus, these data would be consistent with TSP-1 promoting cancer progression in the LNCaP cell model. Migration is another necessary phenotype for cancer progression and a study by

Firlej *et al.* reported that TSP-1-mimetic ABT-510 increases migration in LNCaP cells by 3.5 fold [96]. In the more aggressive cell lines, PC-3 and DU145, I observed that cell proliferation was decreased at higher doses of TSP-1. In contrast, Jin *et al.* reported that DU145 cells showed no change in cell proliferation when TSP-1 was overexpressed by a plasmid clone in these cells [97]. Most studies have, however, shown that TSP-1 is correlated with anti-angiogenic activity and better prognosis [57, 98-100], although some suggest that TSP-1 expression is positively correlated with increased angiogenesis and poorer prognosis in human cancer tissues [101, 102]. My data in PC-3 and DU145 cells are consistent with an anti-tumor role for TSP-1 in PCa.

As CD36 is known to regulate fatty acid uptake in other cell types, it was interesting to note that neither TSP-1 nor neutralizing anti-CD36 antibody affected lipid accumulation in PC-3 and DU145 cells. In a study by Borg *et al.* PEDF was found to regulate systemic fatty acid metabolism by promoting lipolysis in an adipose triglyceride lipase-dependent manner [44] and other studies showed that PEDF induced lipolysis in PCa cells (Doll *et al.* unpublished data). Therefore, since I observed an increase in PEDF, I analyzed the effects of TSP-1 or anti-CD36 antibody treatment on lipolysis. Interestingly, TSP-1 treatment increased lipolytic activity in a dose responsive manner. An increase in lipolytic activity was also seen at higher doses of TSP-1 in PC-3 and LNCaP cells; however, in DU145 cells TSP-1 decreased lipolysis. This suggests that TSP-1, while it does not affect lipid uptake, does affect lipid catabolism in some prostate cells. Lipolytic activity also increased when the CD36 receptor was blocked in RWPE-1, PC-3 cells and a trend was also seen in LNCaP cells. However, this effect was not seen with DU145 PCa cells. PEDF has been shown to influence systemic lipid metabolism in other cell types [44] and therefore, the work presented here, on prostate cells is consistent with the

published literature. Further studies, in which PEDF is blocked, are needed to determine if PEDF is necessary for the increased lipolytic activity in RWPE-1, LNCaP and PC-3 cells.

Disparities observed in TSP-1 and anti-CD36 antibody responses between the prostate cell lines examined in this study are most likely due to the background of genetic mutations. The aggressive cell line PC-3 and DU145 are androgen refractory, as opposed to LNCaP cells, which are androgen sensitive. DU145 cells are known to express the wildtype PTEN protein, a key tumor suppressor gene, as opposed to PC-3 cells, which express mutant protein [103, 104]. PTEN plays a crucial role in cell growth and proliferation. Most human PCa are mutant for this protein, thereby aiding the process of tumorigenesis [105, 106]. Both of these aggressive cell lines, PC-3 and DU145, are also mutant for the well known p53 tumor suppressor gene [82]. The less aggressive PCa cell line, LNCaP, is mutant for PTEN but expresses p53. In addition, while PC-3 and LNCaP cells express wildtype retinoblastoma (Rb), DU145 cells express mutant Rb protein [82]. These genetic differences are summarized in Table 1. Many other mutational differences exist between these cell lines, which could, at least in part, explain the differences in PEDF response to TSP-1 and anti-CD36 antibody treatment that I observed between cell lines in these studies.

Table 1. Genetic changes in PCa cell lines.

| Cell Line | PTEN | p53 | RB |
|------------------|-------------|------------|-----------|
| LNCaP | Mutant | WT | WT |
| PC-3 | Mutant | Mutant | WT |
| DU145 | WT | Mutant | Mutant |

To analyze the effect of TSP-1 on PEDF expression *in vivo*, I used TSP-1 KO mouse prostate tissues. While the PEDF levels were slightly decreased in TSP-1 KO

mice as compared to wildtype mice at 4 month of age, this was not statistically significant and no difference were observed at 6 months of age. As only a few mice were examined per group (3-4), analysis of additional tissues would be appropriate to determine if an actual difference exists. As this TSP-1 KO is a developmental loss model, it is possible that other PEDF regulating molecules are increasing PEDF expression to compensate for the TSP-1 loss, and hence, no differences would be observed in adult tissues.

TSP-1 binding to CD36 receptor in endothelial cells is associated with a well-established signaling cascade which includes several members of the family, src kinase family, fyn, p38 MAPK and JNK (Figure 1). PCa cells are known to express these signaling molecules [68]. Therefore, I analyzed if these mediators are regulated by TSP-1 or neutralizing anti-CD36 antibody treatment in a subset of cell lines PC-3 and DU145. The role of fyn in PCa is unclear. While one study reported loss of fyn expression in PCa [107], another study showed overexpression [108]. My data would support the former study, as no fyn was detected in untreated DU145 or PC-3 cells. It has also been reported that fyn overexpression is linked to aberrant PI3/ Akt pathway signaling leading to anti-apoptotic signals and other hallmarks of cancer (reviewed in [109]). Since TSP-1 treatment increased fyn in my studies the significance of this data in context of the currently published literature is unclear. Since TSP-1 treatment induced fyn expression, while anti-CD36 antibody decreased it, it would appear that fyn regulation is not mediated through a TSP-1 - CD36 pathway. However, this does lead to a new hypothesis that when CD36 receptor is blocked, fyn expression, is downregulated which may also inhibit the PI3/Akt pathway and lead to apoptosis in PCa cells. Future studies should be guided towards testing this hypothesis.

It is reported that both nuclear and total JNK expression is augmented in human malignant prostate epithelium compared to benign tissue (reviewed in [110]). My data would support this as JNK was expressed in DU145 untreated cells. JNK is a mitogen-activated protein kinase known to have pro- and anti-apoptotic functions in PCa, depending on the prostate cell line and treatment conditions used (reviewed in [110]). In DU145 cells, TSP-1 treatment increased JNK expression at all doses. Consistent with these data, Hubner *et al.* established that JNK deficiency in the prostate epithelium may act to induce tumor development in a PTEN knockout model [111]. Other studies suggest that JNK expression is tumor promoting. Kwon *et al.* reported that chemical inhibition of JNK in DU145 cells reduced both cell migration and VEGF expression [112]. It has also been reported that JNK promotes the expression of some proteins responsible for cell invasion and tissue metastasis, such as matrix metalloproteinases (MMPs) -2 and -9 and urokinase-type plasminogen activator [112-114]. In my data, the increase in JNK was associated with decreased proliferation which is consistent with a tumor suppressing activity.

It has been reported that both p38 and its active form p-p38 are overexpressed in human cancerous prostatic epithelium [115-117]. My data would support this as p38 was expressed in untreated PC-3 and DU145 cells. TSP-1 treatment did not change p38 expression in PC-3 cells, as compared to untreated cells. In DU145 cells, p38 expression was elevated at higher doses (5, 10, 20 nM) of TSP-1. Data obtained from blocking the CD36 receptor in DU145 cells shows that p38 expression is diminished (2 µg/ml) or lost (5 µg/ml). In PC-3 cells blocking CD36 receptor shows that p38 expression is decreased at high dose of the antibody. p38 MAPK and its active form, p-p38, have been shown to be overexpressed in human cancerous prostatic epithelium [110]. Huang *et al.* have shown that in PC-3 cells, p38 MAPK activity is essential for TGF-β mediated activation of MMP-2, which aids

in cell invasion [118]. Together, these data suggest that blocking the CD36 receptor in PCa cells may be a novel anti-cancer therapeutic approach.

My data would also suggest that TSP-1 signaling is not acting through inhibitory binding to CD36 since the opposite results the opposite results were obtained with TSP-1 and anti-CD36 antibody treatments. Since all of these molecules have established pro- as well as anti-apoptotic activity in PCa cells, specific studies are needed to determine the roles of these signaling mediators in PEDF expression and lipolytic activity.

CONCLUSION

My hypothesis was that the TSP-1 - CD36 pathway of TSP-1 treatment or blocking the CD36 regulated on PEDF expression in PCa cells and a normal cell prostate epithelial cell line. As PEDF induces lipolysis in PCa cells, I assessed lipolytic activity, cell proliferation and also possible signal mediators that could be involved in TSP-1 - CD36 signaling. In PC-3 cells, I saw an induction in intracellular PEDF expression when the CD36 receptor was blocked. Both TSP-1 treatment and blocking the CD36 receptor in DU145 cells induced PEDF expression. Based on the data collected, a summary model for both PC-3 and DU145 cells is presented (Figure 24). RWPE-1 and LNCaP cells behaved similarly to PC-3 cells. My studies also show that lipid metabolism is positively regulated by exogenous TSP-1 and by blocking the CD36 receptor in PC-3 cells. In contrast, it is negatively regulated in DU145 cells. Of the two pools of PEDF (intracellular and extracellular) intracellular PEDF expression is believed to impact the lipolytic activity in a cell. In my studies, in PC-3 LNCaP and RWPE-1 cells, both TSP-1 or anti-CD36 antibody treatment induced intracellular PEDF and lipolytic activity, suggesting that PEDF may be responsible for lipolytic activity. To test if lipolytic induction is dependent on PEDF expression, future work should test TSP-1 and anti-CD36 antibody treatment in the presence of an siRNA against PEDF. In contrast, in DU145 cells, while TSP-1 treatment or anti-CD36 antibody treatment induced intracellular PEDF expression, lipolysis was inhibited. These data would suggest that, at least in DU145 cells, PEDF does not induce lipolysis.

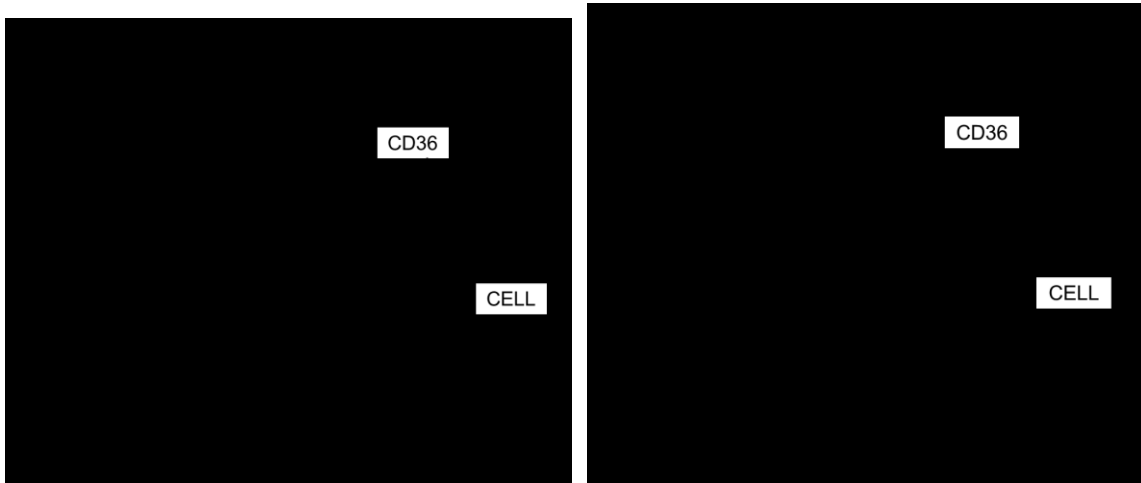


Figure 24. Models of TSP-1 and CD36 receptor signaling in prostate cancer cells. Models based on the data obtained for PC-3 (A) and DU145 (B) cells treated with TSP-1 and neutralizing anti CD36 antibody are presented. TSP-1 treatment induced intracellular PEDF expression in DU145 cells but not in PC-3 cells. Extracellular PEDF expression was inhibited in DU145 but was enhanced in PC-3 cells. Blocking CD36 receptor also increased intracellular and extracellular PEDF expression in both cell lines. Signaling mediators p38 and JNK were in both cell lines with TSP-1 and neutralizing anti-CD36 antibody. Fyn expression was barely detectable PC-3 cells.

To determine if signal mediators, fyn, p38 or JNK, were involved in TSP-1 or CD36 receptor signaling in prostate cells, expression levels were analyzed. Interestingly, both treatments altered levels of p38 MAPK and JNK in both PC-3 and DU145 cells. However, the effects were the opposite for these treatments. TSP-1 increased the expression of these molecules while blocking the CD36 receptor diminishes expression or had no effect. These data are also summarized in Figure 24. The effects of TSP-1 and anti-CD36 antibody treatment on PEDF expression, lipolytic activity and signaling molecules in PC-3, LNCaP and RWPE-1 cells are consistent with my hypothesis. However, the data obtained in DU145 cells is not. To determine if these signaling molecules investigated here are necessary for PEDF expression and/or lipolytic activity, future studies should block each of the signaling kinases, using available chemical inhibitors, with TSP-1 or anti-CD36 antibody treatment.

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