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Thrombospondin-1 Signaling Mechanisms Regulating Pigment Epithelium-Derived Factor Expression and Lipolytic Activity in Prostate Cancer

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by

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ABSTRACT

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Background: Prostate cancer (PCa) is the most common cancer that occurs in men in the United States. To grow, tumors need to induce new blood vessels growth, a process called angiogenesis. Thus, tumors down regulate molecules that inhibit this process. Two such molecules that are down-regulated in PCa are thrombospondin-1 (TSP-1) and pigment epithelium-derived factor (PEDF). Interestingly, both of these proteins also function in regulating lipid metabolism. Our lab has data to suggest that TSP-1 induces PEDF expression and lipid catabolism (lipolysis), the hydrolysis of triglycerides into fatty acids and a glycerol, in PCa cells; however, the molecular pathway of this regulation has not been elucidated. Previous studies in PCa cells have also shown that TSP-1 treatment increases the levels of activated signaling mediators,

JNK and Src kinase. However, it is unclear if induction of JNK or Src is necessary for TSP-1- induced PEDF expression and/or lipolytic activity. In this study, I tested the hypothesis that Src and/or JNK signaling is required for TSP-1- induced PEDF expression and lipolytic activity in PCa cells.

Methods: PCa cell lines, PC-3 (androgen insensitive) and LNCaP (androgen sensitive), and the normal prostate epithelial cell line, RWPE-1, were used in these studies. Cells were treated for 48 h with TSP-1 (5 or 20 nM) with or without chemical inhibitor treatment, either a JNK [SP600125] or Src family kinase [PP2] inhibitor (20 μM). After 48 h, conditioned media (CM) and cell lysate (CL) samples were collected. CM was used to evaluate lipolysis activity via the free glycerol assay. PEDF levels were quanitified in both CM and CL by enzyme linked immunosorbent assay (ELISA) and Western blot analysis.

Results: In RWPE-1, LNCaP, and PC-3 cells, no significant difference was observed in cell proliferation in response to TSP-1 or to either the JNK or Src inhibitor. Viability was decreased in response to TSP-1 in the presence of JNK inhibitor or Src inhibitor in LNCaP cells; whereas, in PC-3 cells, there was a slight increase in viability with TSP-1 in the presence of the JNK inhibitor treatment (P-value <0.010). In RWPE-1 cells, both JNK inhibition and Src inhibition decreased lipolytic activity. Likewise, in the PCa cell lines, lipolytic activity was significantly decreased with either JNK or Src inhibitor treatment as compared to untreated cells (P-value <0.001). In LNCaP cells,

PEDF expression levels were significantly increased with TSP-1 at 5 nM with either JNK or Src inhibitor treatment. However in RWPE-1 CL, PEDF expression was decreased with TSP-1 at 5 nM in the presence of JNK inhibitor treatment.

Conclusion: My data support that TSP-1- induced lipolytic activity is based on the activity of JNK and/or Src family kinase in RWPE-1 cells. The activation of JNK and/or Src family kinase could regulate PEDF expression in RWEP-1 and LNCaP cells. This study suggests that the JNK and/or Src kinase pathways could be a clinically relevant therapeutic target in PCa.

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LIST OF ABBREVIATIONS

PCa, Prostate cancer

PEDF, Pigment epithelium-derived factor

TSP-1, Thrombospondin-1

VEGF, vascular endothelial growth factor

TGF-β1, Transforming growth factor-beta 1

ATGL, Adipose triglyceride lipase

MAPK, Mitogen-activated protein Kinases

JNK, c-Jun amino-terminal kinase

SP600125, Chemical inhibitor of JNK

PP2, Chemical inhibitor of Src family kinases

TNM, Tumor, Nodes, Metastases

PSA, Prostate specific antigen

DRE, Digital rectal exam

TRUS, Transrectal ultrasound-guided

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INTRODUCTION

Prostate cancer

The prostate gland is a part of the male reproductive system. It is a walnut-sized gland located underneath the urinary bladder and in front of the rectum. The main function of the prostate is to secrete fluid that protects and nourishes sperm cells in semen [1]. The prostate gland is composed of four regions: the peripheral zone, central zone, and transition zone, and the anterior fibromuscular stroma [2, 3]. Diseases of the prostate gland include prostatitis, benign prostatic hyperplasia and prostate cancer (PCa), all of which have similar symptoms. The main manifestation of PCa is enlargement of the prostate gland which presses on the urethra. This pressure leads to blockage of urine flow and causes symptoms such as poor urine stream, urine retention, back pain, and hematuria [4].

PCa is an extremely heterogeneous disease, but it is most often an adenocarcinoma or glandular cancer [5]. Approximately 95% of PCa are adenocarcinomas; 70% of these are localized in the peripheral zone, 15-20% in the central zone, and 10-15% in the transition zone [2, 6]. In metastatic PCa, tumor cells have the ability to migrate via the lymphatic system and the bloodstream to other organs, especially to the bone and brain [2]. PCa is the

second leading cause of cancer-related death after lung cancer in men in the United States [2]. According to the National Cancer Institute, in the United States, the number of men who will be diagnosed with PCa in 2014 is approximately 233,000. The estimated number of deaths from PCa is estimated at 29,480 [7]. Even though much progress has been made in understanding PCa, the main causes still remain unclear.

Several factors are believed to be major contributors to PCa development. They include genetic polymorphisms and mutations, increasing age, ethnicity, diet, and obesity [8]. Aging is one of the most significant risk factors increasing the chance of PCa [9], and most cases of PCa occur in older men, over the age of 65 [7]. Many studies have reported that PCa occurs more commonly in men of African and Caribbean descent and less often in other races [10]. This is likely connected to genetic polymorphisms.

In cancer cells, critical genes are mutated or their expression is altered, leading to the cells being abnormal [11]. There are different factors that induce cancer progression, including sustained pro-proliferative signaling, evasion of growth suppressors, apoptosis resistance, replicative immortality, sustained angiogenesis, and tissue invasion and metastasis [12]. Angiogenesis is the growth of new vessels from the existing blood vessels. Tumors need a blood supply to sustain progressive growth, and by inducing angiogenesis the tumor

can grow larger [13]. Oncogenes and tumor suppressor genes are the main two types of genes that regulate these processes. Oncogenes play a causative role in transforming a normal cell to a tumor cell [14]; however, tumor suppressor genes protect and prevent healthy cells from becoming cancerous [15]. When a tumor suppressor gene and/or an oncogene is mutated or altered, this can promote tumor growth.

In addition, another possible risk factor for PCa is a diet that contains red meat and/or is high in fat and/or low in fruits and vegetables [9]. Lastly, obesity is a major risk factor that is associated with disease progression, rather than incidence of disease. Furthermore, obese men who consume milk, cheese, eggs, and meat show a significantly higher risk of lethal PCa compared to lean men [16]. Some general population studies have shown that obesity is highly associated with high-grade PCa at diagnosis [16].

Diagnosis, classification and treatment of PCa

The early stages of PCa often cause minimal or no symptoms. In more advanced stages, symptoms are present, and the PCa can be aggressive. There are three different diagnostic tests that are widely used in conjunction to identify and diagnose PCa. These three tests are the prostate specific antigen (PSA) blood test, the digital rectal examination (DRE), and transrectal ultrasound-guided biopsy (TRUS). The first two are routinely used to screen for PCa, but a

biopsy with histologic examination is necessary to firmly establish a diagnosis of PCa. In DRE, the prostate gland is physically examined to estimate the size of the prostate gland. An elevated PSA level may indicate the presence of PCa; however, the test is not specific, as other diseases of the prostate also elevate PSA. If either the DRE or PSA indicates the possible presence of PCa, a TRUS is used to guide needle biopsies.

Based on histological examination of prostate tissue, as well as other imaging techniques, PCa is divided into different stages and grades. To determine the stage of PCa, the TNM (tumor, node, and metastasis) system is used [17]. Each category has subdivisions to allow precise staging of the cancer. "T" is used to evaluate how much of the prostate is tumorous and also to indicate if the tumor has extended through the prostate capsule and invaded into other nearby structures. The "N" indicates if PCa has migrated to surrounding lymph nodes (proximal lymph nodes). "M" describes if the PCa has metastasized to other parts of the body [17]. The histological grading system for PCa is the Gleason score, which is based on histological examination of prostate tissue stained with hematoxylin and eosin [18]. According to the appearance of the glands and cells in the prostate tissue, a grade of 1 – 5 will be assigned [18]. The most prominent pattern and the second most common pattern are added to give a Gleason score of 2-10 [18]. A score that is 6 or over is considered PCa, and cancers that are scored 7 and above indicates a clinically aggressive cancer.

Additional tests, such as magnetic resonance imaging, computerized tomography scans, and bone scans, may be recommended to identify the stage of cancer progression [19].

If a diagnosis of PCa is confirmed, treatment options are based on the stage and grade of the cancer [18]. If PCa is diagnosed at an early stage, effective treatments are available for patients. These options include surgery (radical prostatectomy), radiotherapy, hormone treatment, immunotherapy treatment, and chemotherapy. In some cases, these treatments are combined together to improve outcomes. However, each treatment can have serious side effects for patients [20]. It is believed that anti-angiogenesis treatments may be effective in treating PCa. Specifically, several novel therapies targeting vascular endothelial growth factor (VEGF), a potent pro-angiogenic factor, and its receptors have improved survival in advanced PCa [21]. Thus, anti-angiogenic drugs are a promising therapy to stop tumor growth.

PCa progression and angiogenesis

Angiogenesis, which is regulated by multiple proteins, can be activated by the presence of pro-angiogenic factors such as VEGF, platelet derived growth factor, basic fibroblast growth factor, and interleukin-8 (IL-8) [13, 22]. On the other hand, angiogenesis can be inhibited by the presence of anti-angiogenic proteins such as pigment endothelium-derived factor (PEDF) and

thrombospondin-1 (TSP-1) [23, 24]. It is ultimately the balance of pro- and anti-angiogenic factors that determines angiogenic activity. The mechanism of production of pro- and anti-angiogenic factors is regulated in normal cells, and the levels of angiogenesis inhibitor proteins are higher than that of inducer proteins. However, in tumors, pro-angiogenic protein levels are increased over that of anti-angiogenic proteins, resulting in induction of angiogenesis, which allows tumor progression. Both TSP-1 and PEDF expression levels are decreased or lost in PCa [25, 26].

Pigment epithelium-derived factor (PEDF) in PCa

PEDF is a 50 kDa multifunctional glycoprotein [27]. Several functions of PEDF are now recognized: a potent inhibitor of angiogenesis, neuroprotection, and an inducer of cell differentiation [28]. PEDF was originally isolated from retinal pigment epithelial cell secretions [27] and belongs to the serine protease inhibitors (serpins) family [27]. The serpins family is composed of two distinctive groups. The structure of these two groups is similar, but one group has a region called the reactive center loop. This region interacts with the specific protease target, but the other group does not have this active region [29]. The first group plays a fundamental role in inflammation, blood coagulation, and extracellular matrix reconstitution [27], while the second group functions in such processes as hormone transport, molecular chaperones, and tumor suppression [29].

PEDF is broadly expressed throughout the body [28], and its function as a potent inhibitor of angiogenesis has been well studied in cancer, and as such, functions as a tumor suppressor. Importantly, lower PEDF levels in cancer tissues correlate with metastasis and poor prognosis in several cancer types, including PCa, pancreatic cancer, hepatic carcinoma and neuroblastoma [30-32]. Emphasizing PEDF's function as a tumor suppressor gene in the prostate, PEDF-deficient mice develop prostate epithelial hyperplasia and increased prostate stromal microvessel density compared to wildtype mice [25]. In addition, the treatment of PCa tumor xenografts with recombinant PEDF decreased stromal vasculature and induced apoptosis [25]. PEDF is also down regulated by androgens in PCa cells [25]. Another study has shown that in human hormone-refractory PCa cells, PEDF inhibits the secretion of IL-8, which is a key effector in the progression of PCa [33].

PEDF in lipid metabolism

In addition to its role as a tumor suppressor, PEDF also has been implicated in regulating lipid metabolism [34]. Lipids play several biological roles, such as energy storage, forming cell membranes, and hormonal regulation [35]. It is believed that alterations in lipid metabolism in cancer cells occur during malignant transformation [36], which may contribute to features of solid tumors, such as growth, proliferation, and invasion [37]. PEDF is also an adipocyte-secreted factor [34]. While some studies report that PEDF is associated with the

development of obesity-related insulin resistance, increasing adipocyte lipolysis, and promoting lipid accumulation in muscle and liver [38, 39], other studies show that PEDF is increased in response to insulin resistance [39]. PEDF is reported to bind to adipose triglyceride lipase (ATGL), which catalyzes the initial step in triglyceride hydrolysis [40]. The main function of ATGL is to remove the first fatty acid from the triglyceride molecule, generating a free fatty acid and a diacylglycerol [41]. With ATGL deficiency in mice, there was increased fat depositions and triglyceride accumulation in nearly every organ [40]. Moreover, PEDF-deficient mice have altered liver lipid metabolism, resulting in hepatic steatosis [42]. Another study in retinal epithelial cells identified that ATGL is a cell surface receptor for PEDF [43]. Other studies have shown that recombinant PEDF co-localizes with ATGL on lipid droplets in hepatocytes and in adipose tissues [42]. PEDF-ATGL interaction was further confirmed by co-immunoprecipitation studies in hepatocyte and human hepatocellular carcinoma cell lines [42]. In an ATGL- deficient mouse model, PEDF induces adipocyte lipolysis and triacylglycerol lipase activity in skeletal muscle and liver in ATGL-dependent manner [44]. Taken together, these results indicate that PEDF is involved in lipid metabolism; however, neither this function nor the molecular pathway has been examined in the prostate.

Thrombospondin-1 (TSP-1) in PCa

The thrombospondin (TSP) family of proteins consists of five multifunctional proteins that interact with a variety of molecules, such as heparin and fibrinogen [45, 46]. Of this family, TSP-1 has been studied intensively. TSP-1 is a large, 420 kDa, homotrimeric molecule and is a matricellular glycoprotein secreted from a variety of normal cells, such as endothelial cells, epithelial cells, fibroblasts, macrophages, and adipocytes [47]. TSP-1 is the only member of the TSP family that has the ability to activate latent transforming growth factor-beta 1 (TGF-β1) [48]. TSP-1 also has an N-terminal globular domain that has the ability to interact with a variety of protein receptors, such as CD36 and CD47 [49]. TSP-1 binding to CD47 induces apoptosis of B-cell chronic lymphocytic leukemia [50] and is responsible for the migratory response of smooth muscle cells [51]. CD36, also known as fatty acid translocase, is a glycosylated protein member of the class B scavenger receptors [52]. CD36 also has several essential functions in fatty acid and glucose metabolism [53]. The interaction between TSP-1 and CD36 is important to activate anti-angiogenesis and inflammation [54]. The interaction between TSP-1 and CD36 induces apoptosis of endothelial cells [55].

TSP-1 has been identified as a functional inhibitor of angiogenesis in normal prostate epithelial cells [26]. Decreased levels of TSP-1 have been reported in PCa cells [26]. These decreased levels, combined with the increased

level of angiogenesis inducers such as vascular endothelial growth factor and fibroblast growth factor in PCa tissue, lead to tumor progression [26]. Some studies have revealed that the regulation of prostate growth is, at least in part, dependent upon TSP-1's activation of TGF-β1, which inhibits prostate epithelial cell proliferation [24]. In TSP-1-deficient mice, microvessel density and epithelial proliferation were increased in the prostate tissue [24]. In the human PCa cell line, LNCaP, the expression level of TSP-1 is decreased in comparison with the PNT 1A cell line, an SV40-immortalized normal human prostate epithelial cell [26].

As stated above, TSP-1 is a matricellular protein secreted from adipose tissue. In several organs, including the kidney, the heart, and adipose tissue, TSP-1 expression levels are increased in animal models of obesity and diabetes [56-59]; however, the role of this increase is not currently understood. Moreover, several studies show that TSP-1 is highly expressed in diabetic and obese patients [59]. Adipose TSP-1 expression has been associated with metabolic dysfunction in obese patients [60]. In previous studies in prostate cells, TSP-1 treatment increased lipolytic activity (Doll et al., unpublished data). However, TSP-1 treated PC-3 cell lines exhibited variable effects on lipolytic activity (Doll et al., unpublished data).

Several reported studies have assessed the correlation between TSP1 and PEDF levels in disease models. In human malignant U251 glioma cells, increased PEDF expression regulates TSP-1 [61]. Another study shows that PEDF treatment increases TSP-1 levels, so that led to decrease human KM12 colon cancer microvessel density, and their expression increased macrophage recruitment [62]. Similarly, other groups show that PEDF and TSP-1 have induced macrophage recruitment into PCa and human melanoma tumors, with increased tumor cell apoptosis [63, 64]. The effects of TSP-1 on PEDF expression in PCa have been examined in several tissue types, as well. TSP-1 treatment increases secreted PEDF levels in RWPE-1, PC-3, DU145, and LNCaP cells (Doll et al., unpublished data). However, intracellular levels of PEDF were increased in LNCaP and PC-3 cells, but decreased in RWPE-1 and DU145 cells, with TSP-1 treatment (Doll et al., unpublished data). In addition, TSP-1 treatment also increased lipolytic activity in PCa cells (Doll et al., unpublished observation). The molecular pathways through which TSP-1 regulates PEDF expression and lipolytic activity in prostate cells is currently uninvestigated.

Signaling mediators, JNK and Fyn, in PCa

Previous studies demonstrated that TSP-1, via signaling molecules c-Jun N-terminal kinase (JNK) and Fyn promotes cell death in endothelial cells [54]. Decreased endothelial cell proliferation and migration in vitro have been reported

after JNK inhibition. Thus, JNK plays a prominent role in regulation of angiogenic activity in endothelial cells [65]. TSP-1, furthermore, is unable to inhibit angiogenesis in the cornea of Fyn null mice [66]. Even though there are many publications describing the effects of various mitogen-activated protein kinase (MAPK) pathways in cancer in general [67], the function of the MAPK pathways in PCa are not clearly understood. Moreover, while the TSP-1 signaling cascade is well established in endothelial cells [54], little is known about its signaling pathways in PCa cells. In preliminary work, TSP-1 treatment induced the expression levels of JNK and Fyn in PCa cells (Doll et al., unpublished data); therefore, in these studies, I investigated if these signaling molecules are necessary for induction of PEDF expression and lipolytic activity in response to TSP-1 treatment. In the PC-3 cell lines, TSP-1 treatment led to increased expression of JNK and Fyn kinases (Doll et al., unpublished data).

JNKs belong to a group of serine/threonine protein kinases known as MAPKs. There are three mammalian genes encoding the JNK family: JNK1, JNK2, and JNK3 [68]. A role for JNKs is well established in proliferation, survival and differentiation of many cell types. The JNK pathway can be activated by ultraviolet radiation [69], cytokines, environmental stresses, and growth factors [70]. JNK expression levels are increased in different types of cancer, such as prostate, breast, lung, and pancreatic [71]. A number of findings show that JNK

expression in PCa is increased in comparison with normal or benign hyperplasia epithelium tissue [72]. Some studies show that JNK may induce PCa metastasis through its ability to regulate cell adhesion, invasion, and migration [73]. In a mouse model of PCa, JNK is increased expression in PCa cells and induces prostate tumorigenicity [74]. On the other hand, chemical inhibition of JNK decreases the activity of cell migration in DU145 PCa cells [73]. Moreover, it has been shown that the activation of JNK signaling pathway is necessary for regulation of cell death in PC-3 cells [75].

Fyn is a member of the Src family of tyrosine protein kinases [76]. Fyn is a 59-kDa protein that functions in the signaling pathway of integrins, which activates the Ras pathway, leading to activation of the MAPK cascade [77]. The biological functions of Fyn are diverse, including cell adhesion, proliferation, ion channel function, and integrin-mediated signaling [77]. Fyn interacts with the cytoplasmic regions of a broad range of surface molecule, such as CD36 and CD44 [78]. In PCa, Fyn is upregulated during cancer progression [79]. It has been indicated that the levels of Fyn and its signaling mediators are increased in LNCaP and PC-3 cell lines [79].

HYPOTHESIS AND SPECIFIC AIMS

PCa is the second leading cause of cancer-related death in men in the United States [7]. The progression of PCa depends on inducing the formation of new blood vessels, a process called angiogenesis. TSP-1 and PEDF are both anti- angiogenic proteins, and their expression is down-regulated during PCa progression [25]. Both of these proteins are multifunctional, and both appear to function in lipid metabolism [42]. Our lab has data to suggest that TSP-1 treatment in PCa cells induces PEDF expression and lipolytic activity (Doll et al., unpublished data), though little is known about the signaling pathway mediating this activity. In other cell types, such as endothelial cells, TSP-1 inhibits endothelial cell proliferation through activation of the signaling molecules JNK and Fyn [54]. In some PCa cell lines, TSP-1 treatment increased the expression level of JNK and Fyn (Doll et al., unpublished data). However, the molecular pathways mediating TSP-1- induced PEDF expression and lipolytic activity in regulation of PCa cells have not been elucidated, and further exploration is needed. It is also unclear if induction of JNK or Fyn is necessary for TSP-1- induced PEDF expression and lipolytic activity. This study examined whether TSP-1 induction of Fyn or JNK signaling is required for TSP-1- induced PEDF expression and lipolytic activity in PCa. The central hypothesis of this study was that ***TSP-1- induced PEDF expression and lipolytic activity is dependent on induction of JNK and/or Fyn signaling in RWPE-1 normal prostate epithelial***

cells and in the PCa cells, LNCaP and PC-3. To examine this hypothesis, I proposed the following specific aims:

Specific Aim 1: Determine if JNK or Fyn signaling is necessary for TSP-1 induced PEDF expression in prostate cells. My working hypothesis for aim 1 was that TSP-1 treatment decreases PEDF expression through JNK and/or Fyn kinase signaling pathways in prostate cell lines. I tested this hypothesis by:

a) Treating cells with TSP-1 with or without a chemical inhibitor of JNK or Fyn and;
b) Measuring PEDF expression levels in both the conditioned media and cell lysates.

Specific Aim 2: Determine if JNK or Fyn signaling is necessary for TSP-1 induced lipolytic activity in prostate cells. My working hypothesis for aim 2 was that blocking JNK and Fyn signaling in PCa cells during TSP-1 treatment inhibits lipolytic activity. I tested this hypothesis by:

a) Collecting an aliquot of conditioned media from cells treated in Aim 1a and;
b) Quantifying lipolytic activity in these samples.

The main objective of this study is to identify the molecular pathway of the effects of TSP-1 treatment on PEDF expression and lipolytic activity in prostate cells. Analyzing the molecular pathways and identifying the mediators of TSP-1 activity in PCa cells may serve as candidates for future therapeutic targets for PCa treatment.

MATERIALS AND METHODS

Cell lines

In this study, three cell lines were used, RWPE-1, LNCAP and PC-3 cells. RWPE-1 cells are an immortalized normal adult human prostatic epithelial cell line from a white male donor, and they were immortalized by transfection with human papillomavirus 18 [80]. PC-3 cells were established from a human prostatic adenocarcinoma metastasis to bone [81], and LNCaP is a PCa cell line derived from a lymph node metastasis of a human PCa [82].

Culture and treatment of cells

RWPE-1 cells were grown in keratinocyte complete media (Gibco, Grand Island, NY, Cat. No. 10724-011) with 1% penicillin / streptomycin (P/S, Cellgro, Manassas, VA, Cat. No. 30-002-CI). PC-3 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Sigma, St. Louis, MO, Cat. No. D5796) with 10% fetal bovine serum (FBS, Sigma, St. Louis, MO, Cat. No. F2442), and 1% P/S. The LNCaP cell line was grown in Roswell Park Memorial Institute (RPMI) media (Sigma, St. Louis, MO, Cat. No. R8758) with 10% FBS and 1% P/S. All cells were incubated at 37°C in 5% CO_2 for propagation.

Cells were cultured to obtain five confluent plates of cells. On day 1 of the experiment, confluent plates of cells were harvested by trypsinization. An aliquot was taken from the trypsinized cells for counting, and cells were centrifuged at 800 x g for eight minutes. The supernatant was aspirated off the cell pellet and discarded. The cell pellet was resuspended in a volume of media to achieve a cell density of 20,000 cells/cm^2 for RWPE-1 and PC-3 cells, and 40,000 cells/cm^2 for LNCaP cells. All cells were plated into 10 cm tissue culture dishes. Therefore, ten dishes were plated for each cell line, as there were 10 treatment groups (Table 1). Each cell line was grown with its own specific growth medium and incubated overnight for cell attachment. On day 2, the growth media was aspirated off, and cells were gently rinsed one time in 5 ml of phosphate buffered saline (PBS, Cellgro, Manassas, VA, Cat. No. 21-031-CV). Serum-free basal media, containing only P/S, was then added, and the cells were incubated for 4 h. This 4 h incubation was to synchronize cells in the cell cycle. During the four hour incubation, treatment medias were prepared in basal media plus 1% P/S only, as described in Table 1. TSP-1 treatments (R&D Systems inc, Minneapolis, MN, Cat. No. 3074-TH) were prepared at 5 or 20 nM, as indicated in Table 1. For the JNK inhibition SP600125 (CALBIOCHEM, Billerica, MA, Cat. No. 420119) was used at 20 μM (Table 1). For Fyn inhibition, a specific inhibitor was not available; therefore, I used the src family kinase inhibitor PP2 (CALBIOCHEM, Billerica, MA, Cat. No. 529573) at 20 μM (Table 1). After the 4 hour incubation, the basal

media were aspirated and the treatment medias were gently added. Cells were then incubated for 48 hours.

Table 1. Treatment groups.

Treatment groups	TSP-1 (nM)	SP600125 (μM)	PP2 (μM)	DMSO (μl)
1. Untreated (negative control)	0	0	0	0
2. TSP-1	5	0	0	0
3. TSP-1	20	0	0	0
4. SP600125 (JNK)	0	20	0	0
5. TSP-1 + SP600125	5	20	0	0
6. TSP-1 + SP600125	20	20	0	0
7. PP2 (Src, Fyn)	0	0	20	0
8. TSP-1 + PP2	5	0	20	0
9. TSP-1 + PP2	20	0	20	0
10. DMSO*	0	0	0	5.1

*DMSO; equal to the largest volume.

Collection of conditioned media

After 48 hours of incubation, the serum-free conditioned media (CM) were collected by treatment group. The CM was then centrifuged at 800 x g for eight minutes at 4ºC to pellet any cells and/or cellular debris. After centrifuging, the CM was transferred to a new conical tube. Aliquots were then taken for the

lipolysis assay, after which protease inhibitors were added to the remaining CM [1X protease inhibitor cocktail (Sigma, St. Louis, MO, Cat. No. P8340) and phenylmethanesulfonyl fluoride (PMSF, 1 µM; Sigma, St. Louis, MO, Cat. No. 93482)]. The samples stored at 4°C until further processing.

Proliferation assay and viability

The treated cells were then trypsinized by adding 1 ml of trypsin reagent to each 10 cm dish (Cellgro, Manassas, VA, Cat. No. 25-053-CL). Trypsinization was stopped by adding 2 ml of growth media, containing serum. A 50 µl aliquot of this cell solution was added to an equal volume of 0.4% trypan blue stock solution (Sigma, St. Louis, MO, Cat. No. T8154). The trypsin/cell solution was incubated for five minutes at room temperature. I then counted total cells and live cells on the Cellometer (Cellometer Auto T4, Bioscience, Nexcelom, Lawrence, MA) per the manufacturer's protocols. Briefly, 20 µl of the cell-trypan blue solution was taken and placed in a Cellometer slide chamber (Nexcelom, Lawrence, MA, Cat. No. SD100) in duplicate. The slide was read for total cell number and viable cell number on the Cellometer for each experimental group. Dead cells incorporated the dye and appeared blue. The living cells appeared colorless and bright. Average cell counts were calculated and used to normalize the lipolysis data.

Quantification of lipolytic activity

Lipolytic activity was determined indirectly by measuring free glycerol levels in the CM. When triglycerides are broken down during lipolysis, three free fatty acids and a glycerol molecule are generated, and the glycerol is secreted from cells. This glycerol can be detected based on an enzymatic reaction using the Free Glycerol Reagent, which produces a colored product that can be quantified by spectrophotometry (Sigma, St. Louis, MO, Cat. No. F6428). The blanks were prepared by adding glycerol reagent alone to a cuvette to adjust the absorbance to zero in a 1.5 mL disposable cuvette. The standards were prepared from the glycerol standard solution (Sigma, St. Louis, MO, Cat. No. G7793) and ranged in concentration from 0.26 to 0.0325 mg/ml.

A serial dilution of glycerol standard was assayed to generate a standard curve. For each of the CM samples, 200 µl of the CM was added to 800 µl of free glycerol reagent. The samples were analyzed on the spectrophotometer (Shimadzu, Kyoto, Tokyo, model No. UV-2501PC). The absorbance at 540 nm was obtained and the concentration based on the standard curve. From the cell count above, free glycerol levels were reported as levels per total cell number. Levels were then compared to untreated as fold-over values.

Cell lysate collection

To collect cell lysate (CL), 1X cell lysis buffer (Cell Signaling, Danvers, MA, Cat. No. 98035) was used with the addition of protease inhibitor cocktail (1X) and PMSF (final [1 µM]). Cells that were trypsinized above were centrifuged at 800 x g for eight minutes after the aliquot was taken for the cell proliferation assays. The supernatant was aspirated and discarded. The cell pellet was resuspended in 350 µl of 1 x cell lysis buffer, and then the cell suspension was incubated on ice for five minutes. I then vortexed the cell mixture for twenty seconds. The CL solution was transferred to a microfuge tube and centrifuged at 14,000 x g at 4°C for ten minutes to pellet the cellular debris. Next, the supernatant was collected into siliconized tubes. Samples were stored at -80°C until use.

CM concentration

For subsequent protein analysis, the CM was concentrated using a Millipore ultra-15 centrifugal filter device (Amicon Ultra Centrifugal Filters, Millipore, Billerka, MA, Cat. No. UFC900324). Using 5 ml of sterile PBS to wet the membrane device, I centrifuged the tube at 4000 x g for 10 minutes at 4°C. The PBS was removed, and then the 5 ml of CM were added and centrifuged at 4000 x g for 30 minutes. Additional spins were performed until almost all of the media

had filtered. The CM was then washed with one to two volumes of PBS to remove the phenol red color. The CM were then concentrated 10 fold, which was approximately a 500 µl volume from 5 ml of CM. Finally, the concentrated CM was transferred to a sterile siliconized microfuge tube and stored it at -80°C until use.

Protein quantification

A Coomassie (Bradford) protein assay was conducted for the quantification of total protein in both the CM and CL samples. In this colorimetric reaction, protein content of a sample is determined by comparison to the development of color in a set of standards that are used to generate a standard curve. The standard solution consisted of a series of known dilutions of bovine serum albumin (BSA, Thermo, Rockford, IL, Cat. No. 23208. The protocol of the Coomassie reagent (Thermo Scientific, Rockford, IL, Cat. No. 1856209) was followed per the manufacturer's instructions. Briefly, 490 µl of Coomassie reagent were added to the tubes. Samples were prepared by adding 10 µl of standard solutions, 10 µl of CM, or 5 µl of CL in addition to 5 µl of PBS. Then, 200 µl of each standard or samples were transferred into the appropriate well of a microplate in duplicate wells. The absorbance at 595 nm was measured on a plate reader (BioTek, Synergy HT). The KC4 program generated a standard curve and calculated the mean concentrations of each sample.

PEDF level detection

In this study, an enzyme-linked immunosorbent assay (ELISA) was employed to quantify the concentration of PEDF levels in both CM and CL samples. The manufacturer's instructions (BioProducts MD, Middletown, MD, Cat. No. PED613) were followed. Briefly, all standards were prepared as a series of known dilutions. Then 100 µl of each CM and CL specimens, or PEDF standard solutions, were pipetted into duplicate wells. Two wells were left empty to serve as blanks. After adding all specimens and standards, the microplate was covered and incubated at 37°C for one hour. After aspirating the solution from the plate, I then washed each well of the microplate five times with 1x wash buffer to remove unbound antigen. Next, 100 µl of PEDF antibody was added to bind to the antigen. After that, the plate was covered and again incubated at 37°C for an hour.

The plate was washed five times to remove the unbound PEDF antibody. After washing, 100 µl of streptavidin peroxidase working solution was pipetted into each well to produce a detectable color in proportion to the PEDF bound in each well. The plate was incubated for 30 minutes at room temperature. After this incubation period, 100 µl of tetramethylbenzidine substrate solution was added to each well to produce a detectable color, and incubated uncovered at room temperature for 30 minutes. To stop the reaction, 100 µl of stop solution, which changes the color from blue to yellow, was pipetted in to each well. The

absorbance at 450 nm in each well was measured on a plate reader (BioTek, Synergy HT). PEDF concentrations were calculated based on the standard curve and compared to untreated sample levels as fold over untreated.

Western blot assay

The Western blot assay was used to visualize and compare the PEDF in CL samples. CL samples were prepared with 2X Laemmeli sample buffer (BIO-RAD, Hercules, CA, Cat. No. 161-0737) in a total volume of 40 µl. Samples were then incubated at 95°C to ensure denaturation and then cell samples were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis [SDS-PAGE; 12% precast acrylamide gel (BIO-RAD, Hercules, CA, Cat. No. 456-1043)]. For a size standard, 10 µl of prestained protein marker (BIO-RAD, Hercules, CA, Cat. No. 161-0374) was used. Gels were run in Tris-glycine-SDS buffer (BIO-RAD, Hercules, CA, Cat. No. 161-0732) at 120 volts for approximately one and a half hours. After proteins were resolved sufficiently on the gels, electrophoresis was stopped, and I then washed the gels with transfer buffer (1X Tris-glycine and 20% methanol) for 10 minutes.

The separated proteins were transferred from the gel, by electroblotting, to a polyvinylidene difluoride (PVDF) membrane (GenHunter Corporation, Nashville, TN, Cat. No. B301-50) at 50 volts for two hours. After the transfer of the proteins from the gels, the membranes were blocked, to prevent nonspecific

binding of the detection antibodies during subsequent steps, at room temperature for an hour. The blocking solution was prepared as follows: 1X Tris buffered saline (TBS, Sigma, St. Louis, MO, Cat. No. T5912) with 0.05% Tween-20 (Sigma, St. Louis, MO, Cat. No. P2287), plus 5% dry milk. The 1X TBS-Tween-20 (TBS-T, 50 ml) solution alone (no dry milk) was used to wash the membranes two times for 10 minutes each.

For PEDF detection, the membranes were hybridized with anti-PEDF antibody (Millipore, Temecula, CA, Cat. No. MAB1059) as the primary antibody. Primary antibody was diluted at 1:1000 in TBS-T and 5% dry milk and incubated at 4°C on a shaker overnight. The following day, membranes were washed three times with 50 ml of TBS-T for 10 minutes each. Secondary antibody, goat anti-mouse IgG horseradish peroxidase (HRP) conjugate (Millipore, Temecula, CA, Cat. No. 12-349), was diluted at 1:2500 in TBS-T plus 5% dry milk. The membranes were then incubated with secondary antibody at room temperature for one hour. The membranes were then washed thrice with 50 ml of TBS-T for 10 minutes each. The membranes were also washed with 50 ml of TBS for ten minutes. The PEDF were detected using a chemiluminescent detection system (Pierce Pico reagents, ThermoScientific, Rockford, IL, Cat. No. 34080) through enzymatic reaction with HRP. The blots were exposed to X-ray film (ThermoScientific, Rockford, IL, Cat. No. 34093) to obtain an autoradiogram for a permanent record.

Probed membranes were stripped using boiling 1% SDS at room temperature for two hours. The stripped membranes were washed and blocked before re-probing. For a protein loading control, GAPDH expression was detected in the stripped membranes by conducting the same Western blot protocol as described above. Anti-GAPDH primary antibody was used (Cell Signaling, Danvers, MA, Cat. No. 3683S). Anti-rabbit IgG, HRP linked antibody (Cell Signaling, Danvers, MA, Cat. No. 7074S) was utilized as secondary antibody.

Statistical Analysis

All the experiments were repeated at least twice. Data for each kinase inhibitor were graphed and analyzed separately; however, the controls used (untreated, TSP-1, and DMSO treatments) were the same. Student t-test and ANOVA analysis were performed to test if significant results were obtained in these experiments. ANOVA with Bonferroni post-hoc test was used to compare dose responsiveness. Student t-test was employed for pairwise comparisons where appropriate. PEDF levels and free glycerol levels in TSP-1 treated cells were compared to untreated cells and to TSP-1 with chemical inhibitors of JNK and Fyn. Differences between experimental groups were considered statistically significance if the P value was ≤ 0.05.

RESULTS

The effects of TSP-1 treatment with or without kinase inhibitor treatment on the proliferation and viability of normal prostate epithelial cells and PCa cells

TSP-1 suppresses angiogenic activity both in *in vivo* and *in vitro* experiments and slows the growth of tumors by inhibiting angiogenesis [83]. In TSP-1-deficient mice, epithelial proliferation was increased in the prostate [24]. In endothelial cells, TSP-1 promotes cell death by activating signaling molecules JNK and Fyn [54]. To investigate whether Src or JNK signaling was necessary for TSP-1 signaling, I treated normal prostate epithelial cells (RWPE-1) and PCa cells (LNCaP and PC-3) with 5 or 20 nM TSP-1 in the presence or absence of a JNK inhibitor or a Src family kinase inhibitor (20 μM). The JNK pathway was inhibited using SP600125, a JNK1/2 specific inhibitor. A specific Fyn kinase inhibitor was not available; therefore, I used PP2, a chemical inhibitor of Src family kinases. I then assessed total cell numbers and viable cell numbers on a Cellometer.

As shown in Figure 1A, no significant differences were observed in RWPE-1 cell proliferation between groups. Likewise, no differences in proliferation were observed in the PCa cells, PC-3 and LNCaP (Figure 1B and 1C). With regards to

cell viability, no significant difference was observed between groups in RWPE-1 cells (Figure 2A). As shown in Figure 2B, in PC-3 cells, the only treatment group that had a slight, but significant, effect on cell viability was the TSP-1 (5 nM) with JNK inhibitor, SP600125, treatment as compared to either TSP-1 at 5 nM alone (P-value <0.010) or JNK inhibitor alone (P <0.044). In LNCaP cells, a decrease in cell viability was observed with TSP-1, 20 nM with JNK inhibitor, SP600125, treatment compared to TSP-1, 20nM alone (P-value <0.012), but not as compared to JNK inhibitor alone (Figure 2C). No other differences in viability were observed in these cells.

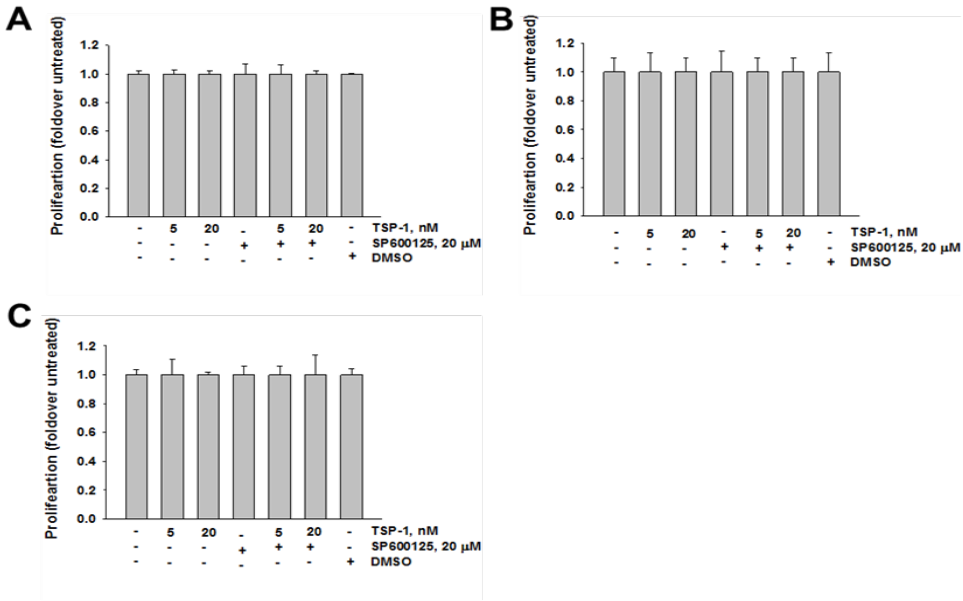

Figure 1. TSP-1 treatment and JNK inhibitor, SP600125, treatment do not affect proliferation of RWPE-1, PC-3 or LNCaP cells. Both RWPE-1 **(A)** and PC-3 **(B)** were plated at 20,000 cells/cm^2, and LNCaP cells **(C)** were plated at 40,000 cells/cm^2 in tissue culture dishes. All cells were incubated overnight. Cells were treated with TSP-1 with or without a chemical inhibitor of JNK (SP600125) and incubated for 48 hours. Cells were collected by trypsinization, and total cell numbers were counted on a Cellometer. Data were presented as fold over the untreated control group. DMSO was the solvent for the JNK inhibitor. Controls were the untreated cells, TSP-1, and DMSO treatments. The experiment was repeated at least twice with similar results.

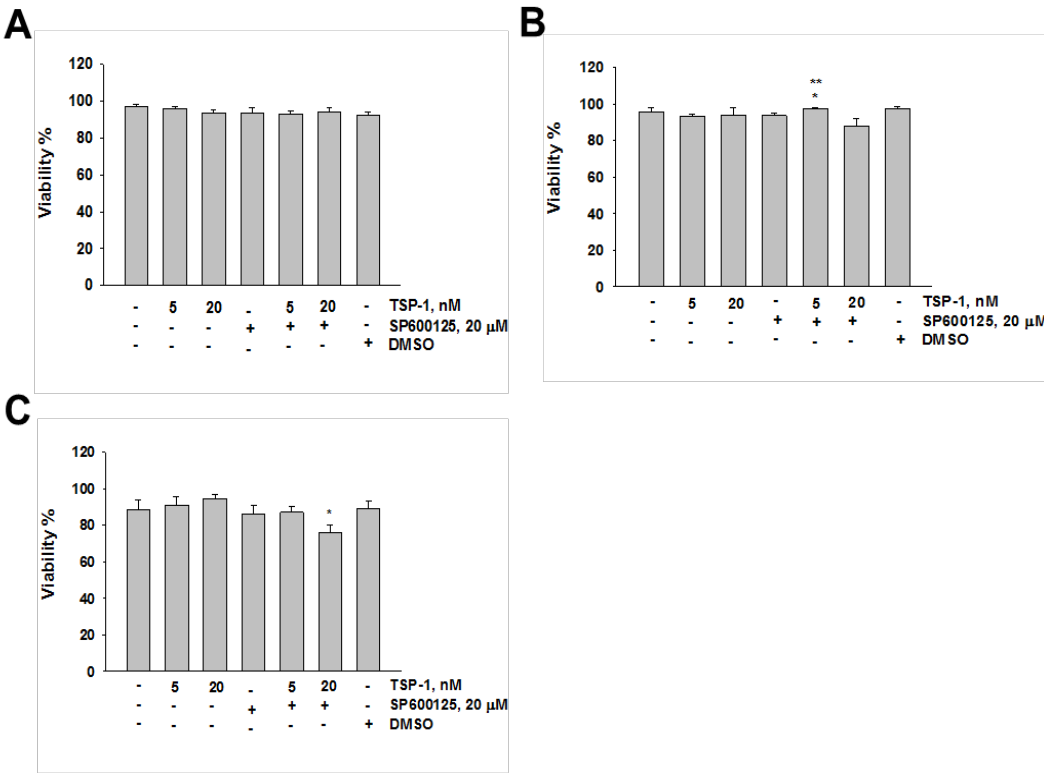

Figure 2. The effect of TSP-1 treatment and JNK inhibitor, SP600125, treatment on viability in RWPE-1, PC-3, or LNCaP cells. RWPE-1 and PC-3 cells were plated at 20,000 cells/cm^2, and LNCaP cells were plated at 40,000 cells/cm^2 in tissue culture dishes and incubated overnight. Cells were treated with TSP-1 with or without a chemical inhibitor of JNK (SP600125) and incubated for 48 hours. Cells were collected by trypsinization, and viability was measured on a Cellometer. Data are presented as percentages of viable cells (viable cells / total cells) in RWPE-1 **(A),** PC-3 **(B),** and LNCaP **(C)** cells. In **(B),** *P-value <0.010 compared to TSP-1, 5nM; **P-value <0.044 compared to JNK inhibitor. In **(C),** *P-value <0.012 compared to TSP-1, 20nM. DMSO was the solvent for the JNK inhibitor. Controls were the untreated cells, TSP-1, and DMSO treatments. This is the result of two independent experiments.

With TSP-1 with or without Src kinase family inhibitor, PP2, treatment, no significant differences were observed in RWPE-1 proliferation (Figure 3A). Likewise, these treatments did not change proliferation in PC-3 and LNCaP cells (Figure 3B and 3C). As shown in Figure 4A, PP2 treatment alone decreased RWPE-1 cell viability as compared to the untreated group (P-value <0.02).

Similarly, there was a decrease in viability with the TSP-1, 20 nM, with Src inhibitor, PP2, as compared to TSP-1, 20 nM alone (P-value <0.001); however, this difference was not significantly different from PP2 treatment alone (Figure 4A). In PC-3 cells, there was no significant change in viability with any of the treatments (Figure 4B). In LNCaP cells, PP2 treatment alone, and with TSP-1 treatment, decreased viability although only the TSP-1 20 nM with PP2 treatment reached statistical significance as compared to TSP-1 at 20 nM alone (Figure 4C; P-value <0.031).

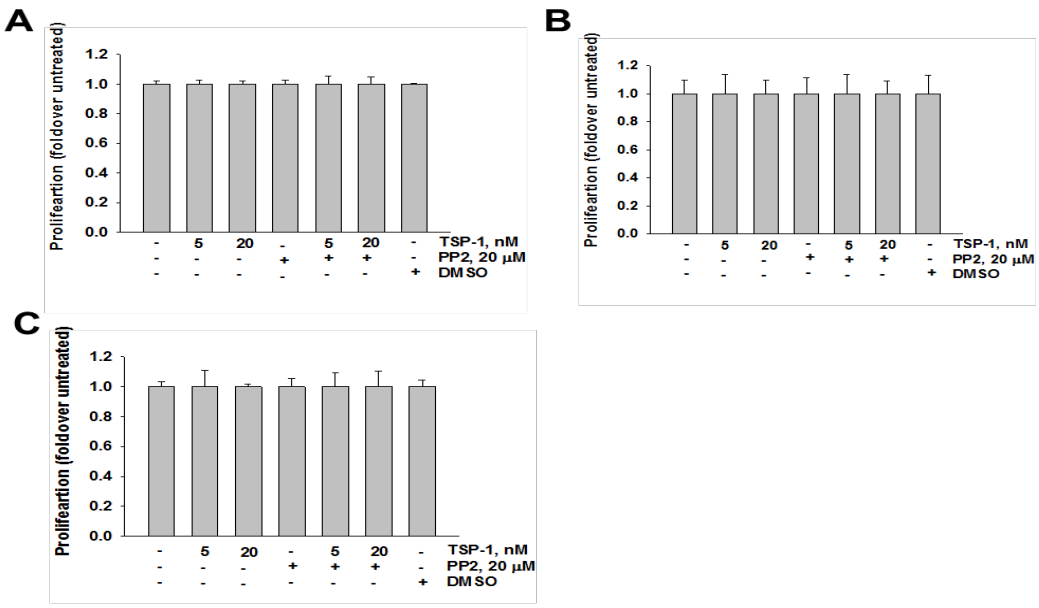

Figure 3 TSP-1 treatment and Src family kinase inhibitor, PP2, treatment do not affect proliferation of RWPE-1, PC-3 or LNCaP cells. Both RWPE-1 **(A)** and PC-3 **(B)** were plated at 20,000 cells/cm^2, and LNCaP cells **(C)** were plated at 40,000 cells/cm^2 in tissue culture dishes. All cells were incubated overnight. Cells were then treated with TSP-1 with or without a chemical inhibitor of Src family kinases (PP2) and incubated for 48 hours. Cells were collected by trypsinization, and total cell numbers were counted on a Cellometer. Data are presented as fold over the untreated control group. DMSO control was the equivalent volume to PP2 dose (largest volume). Controls, which were the untreated cells, TSP-1, and DMSO treatments, were the same controls as presented in Figure 1. The experiment was repeated at least twice with similar results.

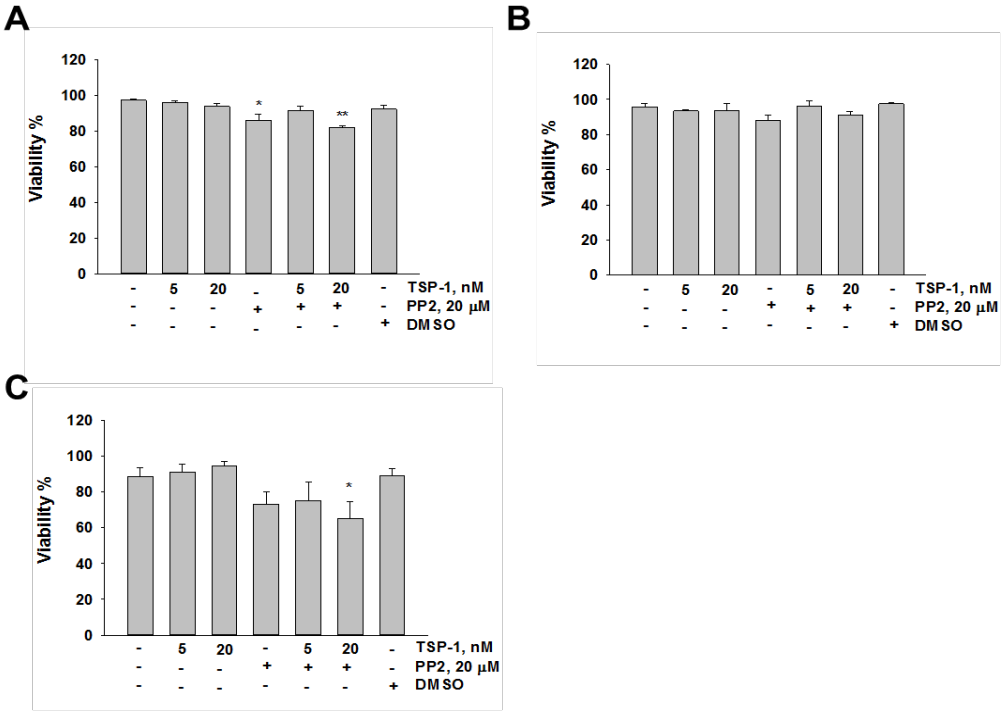

Figure 4. The effect of TSP-1 treatment and Src family kinase inhibitor, PP2, treatment on viability of RWPE-1, PC-3, or LNCaP cells. RWPE-1 **(A)** and PC-3 **(B)** cells were plated at 20,000 cells/cm^2, and LNCaP cells **(C)** were plated at 40,000 cells/cm^2 in tissue culture dishes. All cells were incubated overnight. Cells were treated with TSP-1 in the presence or absence of chemical inhibitor of Src family kinases (PP2) and incubated for 48 hours. Cells were collected by trypsinization, and viability was measured on a Cellometer. Data were presented as percentages of viable cells (viable cells / total cells). In **(A)**, *P-value <0.02 compared to untreated group; **P-value <0.001 compared to TSP-1, 20nM group. In **(B)**, there was no significant difference between groups. In **(C)**, *P-value <0.031 compared to TSP-1, 20nM. DMSO control was the equivalent volume to PP2 dose (largest volume). Controls, which were the untreated cells, TSP-1, and DMSO treatment, were the same controls as presented in Figure 2. This is the result of two independent studies.

The effects of TSP-1 with or without JNK inhibitor on PEDF protein levels in conditioned media (CM) and cell lysates (CL) of normal prostate epithelial cells or PCa cell lines

Both TSP-1 and PEDF expression are decreased in PCa tissues [25, 26]. In human and rat prostate tumors, decreased PEDF levels correlate with a metastatic phenotype [26]. Our lab has preliminary data showing that TSP-1 treatment at 5 or 20 nM induced PEDF expression in PCa cells (Doll et al., unpublished data). In the present study, I asked if this TSP-1-induced PEDF expression was dependent on JNK or Src kinase signaling. PEDF levels were assessed in both CM and CL samples by ELISA. As shown in Figure 5A, TSP-1 treatment alone did not alter secreted PEDF levels in RWPE-1 cells, while the JNK inhibitor, SP600125, alone significantly diminished secreted PEDF expression compared to untreated group (P-value <0.001). TSP-1 treatment appeared to modestly block SP600125's effect on PEDF levels. With JNK inhibitor, SP600125, with TSP-1 treatment, PEDF levels were decreased compared to each TSP-1 dose alone (P-value <0.001); however, compared to JNK inhibitor alone, PEDF levels were increased (Figure 5A; P-value <0.001). In this experiment, the DMSO control slightly increased PEDF expression compared to untreated group, and this increase was statistically significant (Figure 5A; P-value <0.001). In LNCaP cells, only the 5 nM TSP-1 treatment with JNK inhibitor, SP600125, significantly

decreased secreted PEDF expression as compared to TSP-1, 5 nM alone (Figure 5B; P-value <0.009).

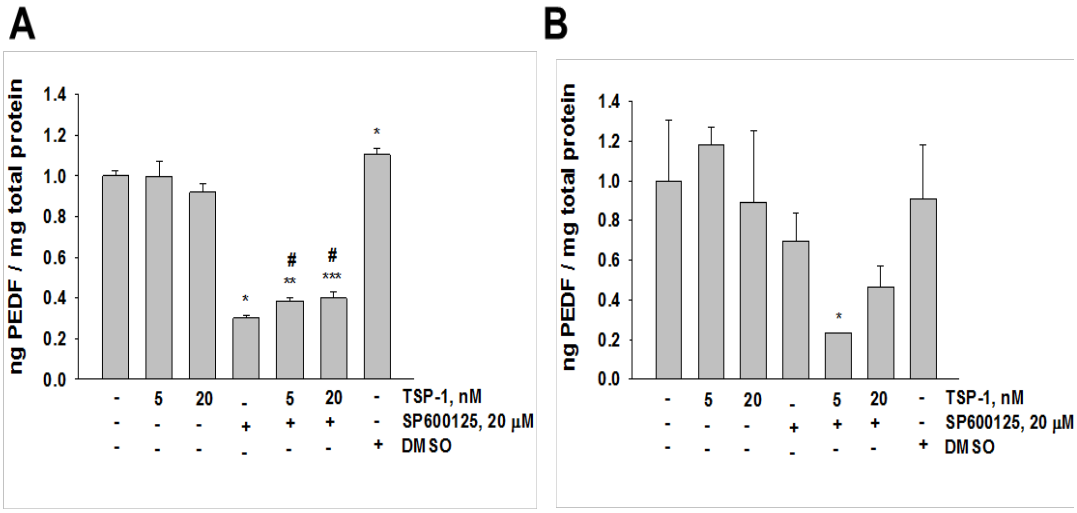

Figure 5. The effect of TSP-1 and JNK inhibitor, SP600125, treatment on PEDF expression in conditioned media (CM) in RWPE-1 or LNCaP cells. RWPE-1 **(A)** cells were plated at 20,000 cells/cm^2, and LNCaP cells **(B)** were plated at 40,000 cells/cm^2 in tissue culture dishes. All cells were incubated overnight. Cells were then treated with TSP-1 in the presence or absence of the JNK chemical inhibitor, SP600125, and incubated for 48 hours. Serum-free CM was then collected and PEDF quantified by ELISA. Data are presented as fold over the untreated control group. In **(A)**, *P-value <0.001 compared to untreated; **P-value <0.001 compared to TSP-1, 5nM; ***P-value <0.001 compared to TSP-1, 20nM; #P-value <0.011 compared to JNK group. In **(B)**, *P-value <0.009 compared to TSP-1, 5nM group. DMSO control was the equivalent volume to PP2 dose (largest volume). Controls were the untreated cells, TSP-1, and DMSO treatments. For RWPE-1, the experiment was repeated at least twice with similar results. For LNCaP, this is the result of one experiment.

Intracellular PEDF expression was evaluated by ELISA in CL samples collected from RWPE-1 and LNCaP cells. In RWPE-1 cells, no significant changes were observed in the levels of PEDF protein in CL of TSP-1 treatment alone or the JNK inhibitor alone (Figure 6A). Interestingly, SP600125 treatment alone did not alter PEDF levels; however, decreased PEDF expression was observed with 5 nM TSP-1 with SP600125 treatment as compared to JNK inhibitor alone (Figure

6A; P-value <0.011). In this cell line, the DMSO control moderately decreased PEDF expression compared to the untreated group (Figure 6A; P-value <0.013). In LNCaP cells, TSP-1 treatment alone did not significantly alter PEDF levels, although a modest decrease was observed at the 20 nM dose (Figure 6B; P-value <0.081). While there was a slight increase in intracellular PEDF levels with SP600125 alone, this was not statistically significant (Figure 6B; P-value <0.183). However, with SP600125 with the 5 nM TSP-1 treatment, the increase was statistically significant as compared to 5 nM TSP-1 treatment alone (P-value <0.016).

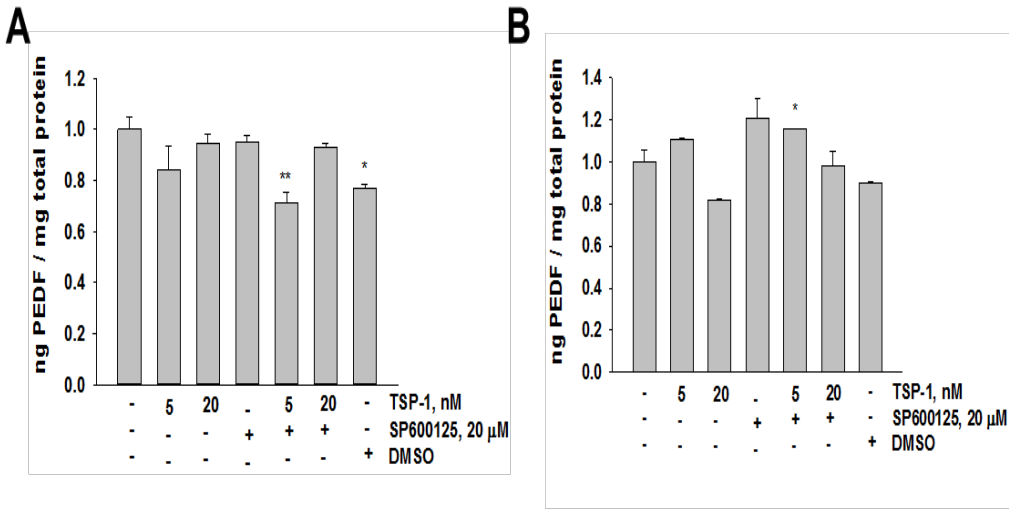

Figure 6. The effect of TSP-1 and JNK inhibitor, SP600125, treatment on PEDF expression in cell lysate (CL) in RWPE-1 and LNCaP cells. RWPE-1 **(A)** cells were plated at 20,000 cells/cm^2, and LNCaP cells **(B)** were plated at 40,000 cells/cm^2 in tissue culture dishes. All cells were incubated overnight. Cells were then treated with TSP-1 with or without a chemical inhibitor of JNK, SP600125, and incubated for 48 hours. Cells were trypsinized, and cell lysate collected. Cellular PEDF levels were assessed by ELISA. Data were presented as fold over the untreated control group. In **(A)**, *P-value <0.004 compared to untreated; **P-value <0.002 compared to JNK inhibitor group. In **(B)**, *P-value <0.016 compared to TSP-1, 5nM. DMSO control was the equivalent volume to PP2 dose (largest volume). Controls were the untreated cells, TSP-1, and DMSO treatments. Two independent studies of RWPE-1 are presented. For LNCaP, this is the result of one experiment.

The effects of TSP-1 with or without Src family kinase inhibitor on PEDF protein levels in conditioned media (CM) and cell lysates (CL) of normal prostate epithelial cells or PCa cell lines

In this study, two independent experiments on RWPE-1 cells showed differing results. In the first experiment, the data showed that an increase in secreted PEDF protein in the CM of the PP2 inhibitor alone as compared to untreated cells (Figure 7A; P-value <0.002). TSP-1 at 20 nM with PP2 inhibitor treatment significantly increased secreted PEDF levels as compared to TSP-1 at 20 nM (P-value <0.001). Nonetheless, a decrease in secreted PEDF levels was detected with TSP-1 at 5 nM with PP2 treatment as compared to either 5 nM TSP-1 group (P-value <0.001) or PP2 inhibitor group (P-value <0.002). There were no effects on PEDF levels with TSP-1 treatment alone as compared to the untreated cells (Figure 7A; P-value <0.182). However, in a second experiment using RWPE-1 cells, the opposite results were obtained. The 5 nM TSP-1 treatment significantly increased secreted PEDF levels compared to the untreated group (Figure 7B; P-value <0.001). However, 20 nM TSP-1 decreased extracellular PEDF protein levels as compared to untreated group (P-value <0.001). Moreover, PP2 treatment alone or with 5 or 20 nM TSP-1 significantly decreased PEDF levels in RWPE-1 CM as compared to related groups (Figure 7B; P-value <0.001). The DMSO control slightly induced PEDF levels compared to

untreated cells (P-value <0.001). Based on these results, additional experiments are needed to clarify the effect of TSP-1 on PEDF expression.

In LNCaP cells, in one experiment, the 5 nM TSP-1 treatment with PP2 inhibitor treatment increased secreted PEDF levels as compared to 5 nM TSP-1 alone (Figure 7C; P-value < 0.020). In addition, secreted PEDF protein levels were increased with PP2 inhibitor alone or with 20 nM TSP-1, yet neither was statistically significant. TSP-1 treatment alone in CM of LNCaP cells did not alter PEDF expression. This experiment needs to be repeated to confirm results.

In RWPE-1 cells, intracellular PEDF levels were not changed with TSP-1 treatment as compared to untreated cells (Figure 8A; P-value <0.411). Similarly, no significant differences were observed with the 5 or 20 nM TSP-1 treatment with PP2 inhibitor treatment as compared to the TSP-1 at 5 or 20 nM groups and the PP2 group. The DMSO treatment slightly decreased PEDF protein levels in CL of RWPE-1 (P-value <0.004). In LNCaP cells, although increased PEDF levels were detected with PP2 inhibitor alone or with 5 or 20 nM TSP-1 as compared to related groups, no statistically significant differences were observed (Figure 8B). The 5 nM TSP-1 dose increased PEDF expression in CL of LNCaP cells, but it was not statistically significant (Figure 8B).

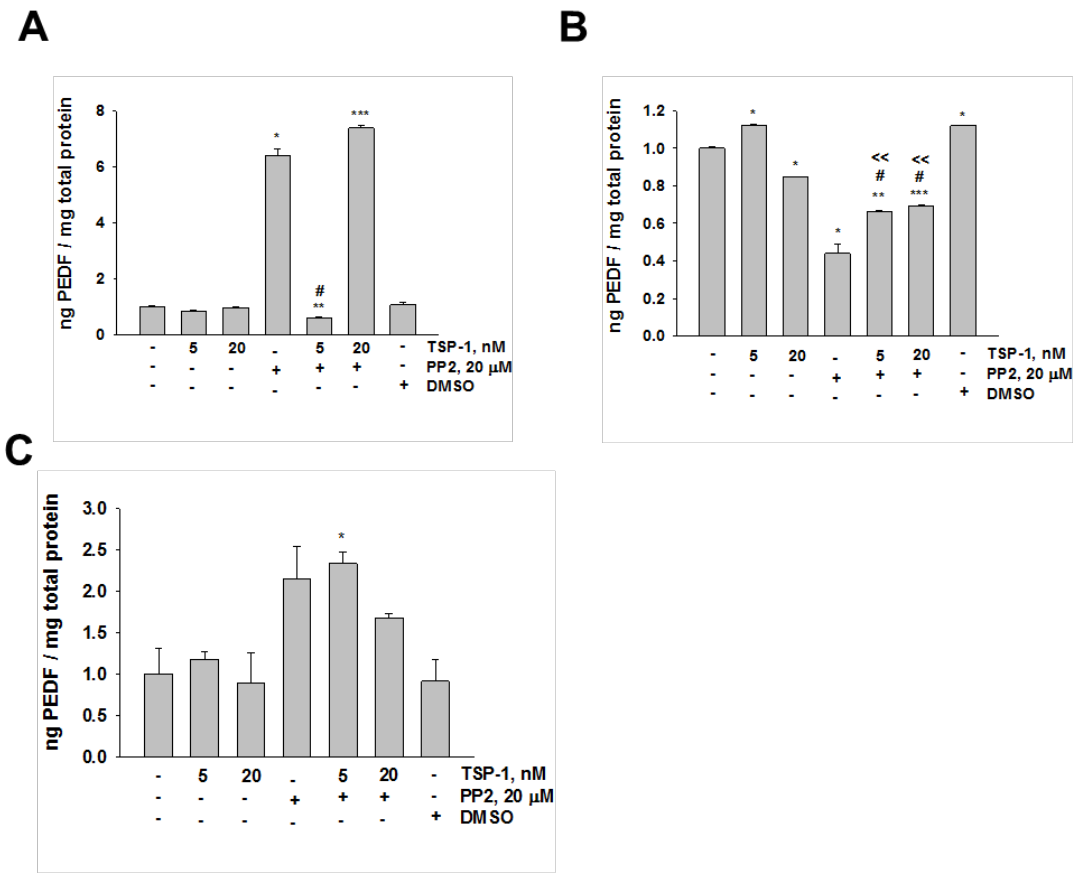

Figure 7. The effect of TSP-1 and Src kinase family inhibitor, PP2, treatment on PEDF expression in CM in RWPE-1 and LNCaP cells. RWPE-1 **(A** and **B)** cells were plated at 20,000 cells/cm^2, and LNCaP cells **(C)** were plated at 40,000 cells/cm^2 in tissue culture dishes. All cells were incubated overnight. Cells were then treated with TSP-1 with or without a chemical inhibitor of Src family kinase, PP2, and incubated for 48 hours. Serum-free CM was then collected, concentrated and protein content quantified. PEDF levels were measured by ELISA. Data were presented as fold over the untreated control group. Significant differences were found between treatment groups. In **(A** and **B)**, *P-value <0.001 compared to untreated; **P-value <0.001 compared to TSP-1, 5nM group; ***P-value <0.001 compared to TSP-1, 20nM; #P-value <0.002 compared to PP2 group;<<P-value <0.001 compared to DMSO control group. In **(C)**, *P-value <0.020 compared to TSP-1, 5nM. DMSO control was the equivalent volume to PP2 dose (largest volume). Controls, which were the untreated cells, TSP-1, and DMSO treatments, were the same as presented in Figure 5. Two independent studies of RWPE-1 are presented. For LNCaP, this is the result of one experiment.

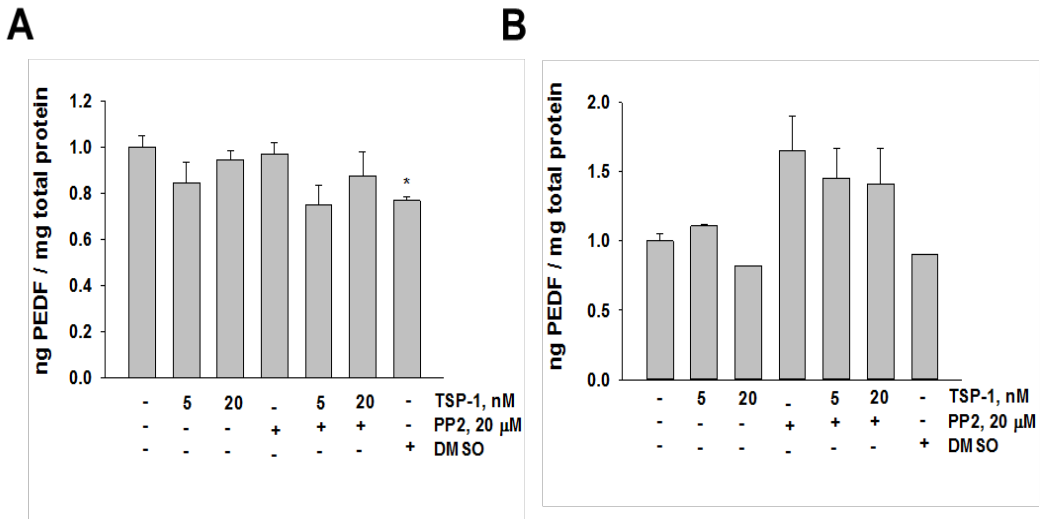

Figure 8. The effect of TSP-1 and Src kinase family inhibitor, PP2, treatment on PEDF expression in CL in RWPE-1 and LNCaP cells. RWPE-1 **(A)** cells were plated at 20,000 cells/cm^2, and LNCaP cells **(B)** were plated at 40,000 cells/cm^2 in tissue culture dishes. All cells were incubated overnight. Cells were then treated with TSP-1 in the presence or absence of Src family kinase inhibitor, PP2, and incubated for 48 hours. Cells were trypsinized, and cell lysates were collected and the total protein content quantified. PEDF levels were assessed by ELISA. Data are presented as fold over the untreated control group. In **(A)**, *P-value <0.004 compared to untreated. DMSO control was the equivalent volume to PP2 dose (largest volume). Controls, which were the untreated cells, TSP-1, and DMSO treatments, were the same as presented in Figure 6. Two independent studies of RWPE-1 are presented. For LNCaP, this is the result of one experiment.

To visualize the PEDF in the CL samples and to confirm the molecular weight of the PEDF, Western blot assays were performed on LNCaP and PC-3 CL samples. As depicted in Figure 9, PEDF expression was detected in untreated samples and experimental samples. Membranes were stripped and re-probed with GAPDH as a control for equal protein loading (Figure 9).. PEDF protein levels in the CL of LNCaP cells treated with SP600125 inhibitor alone or with 5 or 20 nM TSP-1 increased in ELISA while decreased PEDF expression was observed in Western blot assay. No differences were observed with PP2 inhibitor alone or with 5 or 20 nM TSP-1 in these assays. Intracellular PEDF expression in PC-3 cell

lines was also detected. As shown in Figure 10, PEDF expression was detected in untreated samples, TSP-1 treatment alone samples, and DMSO treatment samples. In addition, PEDF expression was identified in TSP-1 in the presence of SP600125 or PP2 samples. GAPDH was used as a control for equal protein loading.

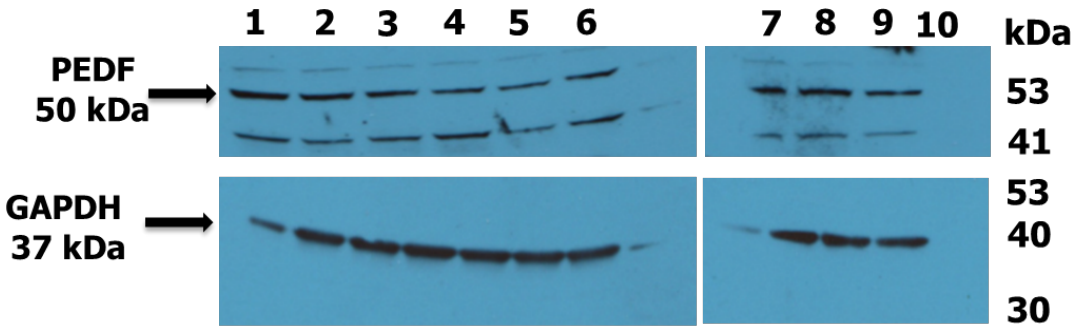

Figure 9. The effect of TSP-1 and JNK or Src family kinase inhibitor treatment on PEDF expression in CL samples in LNCaP cells. LNCaP cells were plated at 40,000 cells/cm^2 in tissue culture dishes and were incubated overnight. Cells were treated with TSP-1 with or without specific JNK inhibitor (SP600125) or a Src family kinase inhibitor (PP2). CL samples were separated on SDS-PAGE gels (12%). The separated proteins were transferred to PVDF membrane by electroblotting. Membranes were probed with anti-PEDF antibody, stripped and reprobed with GAPDH antibody, which served as a control for protein loading. Lanes are as follows: 1, Untreated sample; 2, 5 nM TSP-1; 3, 20 nM TSP-1; 4, 20 μM JNK inhibitor; 5, 5 nM TSP-1 + 20 μM JNK inhibitor; 6, 20 nM TSP-1 + 20 μM JNK inhibitor; 7, 20 μM Src inhibitor; 8, 5 nM TSP-1 + 20 μM Src inhibitor; 9, 20 nM TSP-1 + 20 μM Src inhibitor; 10, DMSO control.

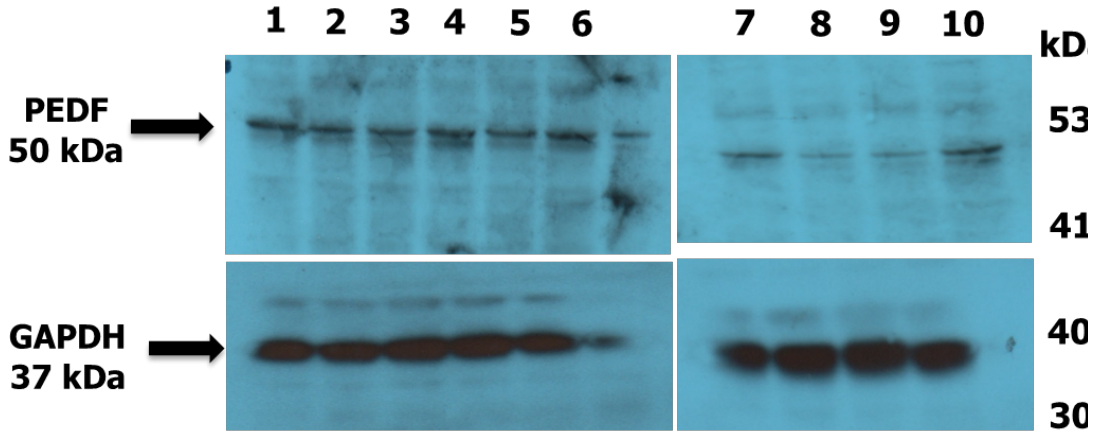

Figure 10. The effect of TSP-1 and JNK or Src family kinase inhibitor treatment on PEDF expression in CL samples in PC-3 cells. PC-3 cells were plated at 20,000 cells/cm^2 in tissue culture dishes and were incubated overnight. Cells were treated with TSP-1 with or without a specific JNK inhibitor (SP600125) or a Src family kinase inhibitor (PP2). CL samples were separated on SDS-PAGE gels (12%). The separated proteins were transferred to PVDF membrane by electroblotting. Membranes were probed with anti-PEDF antibody, stripped and reprobed with GAPDH antibody, which served as a control for protein loading. Lanes are as follows: 1, Untreated sample; 2, 5 nM TSP-1; 3, 20 nM TSP-1; 4, 20 μM JNK inhibitor; 5, 5 nM TSP-1 + 20 μM JNK inhibitor; 6, 20 nM TSP-1 + 20 μM JNK inhibitor; 7, 20 μM Src inhibitor; 8, 5 nM TSP-1 + 20 μM Src inhibitor; 9, 20 nM TSP-1 + 20 μM Src inhibitor; 10, DMSO control.

The effect of TSP-1 with or without JNK inhibitor on the lipolytic activity of normal prostate epithelial cells and PCa cells

Preliminary studies suggest that TSP-1 treatment plays a role in PCa cell lipid metabolism (Doll et al., unpublished data). Previous work has also shown that PEDF regulates lipid metabolism in PCa cells (Doll et al., unpublished data). Therefore, the potential alteration of lipolytic activity in the CM of RWPE-1, LNCaP, and PC-3 cells treated with 5 or 20 nM TSP-1 with or without SP600125 or PP2 inhibitor treatment was evaluated by a quantification of secreted glycerol.

In RWPE-1 cell lines, the 5 nM TSP-1 treatment increased lipolytic activity compared to untreated cells (P-value <0.015) while there was no statistically significant difference in lipolysis with 20 nM TSP-1 (Figure 11A). However, SP600125 treatment alone decreased lipolytic activity, but this was not statistically significant in comparison to untreated group (Figure 11A; P-value <0.098). A significant decrease in lipolytic activity of RWPE-1 was detected with the 5 nM TSP-1 with SP600125 inhibitor treatment as compared to TSP-1 at 5nM (Figure 11A; P-value <0.023). Similarly, 20 nM TSP-1 in the presence of JNK inhibitor significantly reduced lipolysis compared to 20 nM TSP-1 (P-value <0.027). As shown in Figure 11B, 5 nM TSP-1 treatment increased lipolytic activity of PC-3 cells, but this increase was not a statistically significant difference. However, a significant decrease in lipolysis was observed with TSP-1 at 5nM treatment with SP600125 inhibitor treatment as compared to TSP-1, 5nM (Figure 11B; P-value <0.010).

In LNCaP cells, in two combined independent experiments, the 5 or 20 nM TSP-1 treatment alone reduced liplolysis, yet this reduction was not statistically significant (Figure 11C). However, an increase in lipolytic activity in LNCaP was observed with SP600125 inhibitor treatment alone, or with the 5 or 20 nM TSP-1, treatment, but again was not significantly different (Figure 11C). In a second independent experiment with LNCaP cells, the results showed that lipolysis was

induced with the 5 or 20 nM TSP-1 treatment, but no significant differences in lipolysis were observed in contrast to the untreated group (Figure 11D). Furthermore, SP600125 inhibitor treatment alone decreased lipolytic activity compared to the untreated group (Figure 11D; $P < 0.001$). Interestingly, increased lipolytic activity was detected with 5 or 20 nM TSP-1 treatment with JNK inhibitor as compared to the JNK inhibitor group (Figure 11D; P-value <0.001). The DMSO control significantly decreased lipolytic activity compared to untreated group (Figure 11D; P-value <0.001).

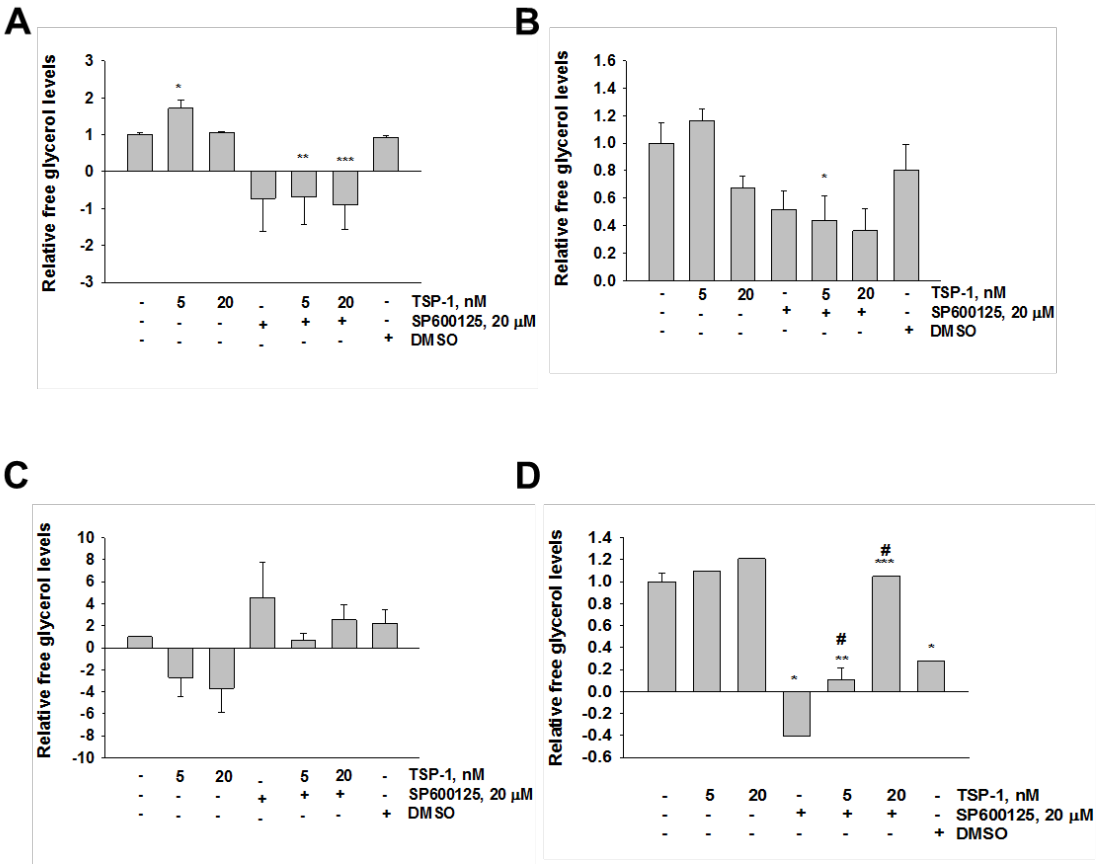

Figure 11. The effect of TSP-1 with a chemical inhibitor of JNK kinase on lipolytic activity in RWPE-1, PC-3 and LNCaP cells. Both RWPE-1 **(A)** and PC-3 **(B)** were plated at 20,000 cells/cm^2, and LNCaP cells **(C** and **D)** were plated at 40,000 cells/cm^2 in tissue culture dishes. All cells were incubated overnight. Cells were then treated with TSP-1 with or without chemical inhibitor of signaling mediator JNK, SP600125, and incubated for 48 hours. Serum-free conditioned media was then collected and lipolytic activity quantified using the free glycerol assay. Data were normalized to total cell count and compared as fold over values. In **(A)**, *P-value <0.015 compared to untreated group; **P-value <0.023 compared to TSP-1, 5nM group; ***P-value <0.027 compared to TSP-1, 20nM group. In **(B)**, P-value <0.010 compared to TSP-1, 5nM. No statistical differences were found in **(C)** cells. In **(D)**, *P-value <0.001 compared to untreated group; **P-value <0.001 compared to TSP-1, 5nM group; ***P-value <0.001 compared to TSP-1, 20nM group; #P-value <0.001 compared to JNK inhibitor group. DMSO control was an equivalent volume to PP2 dose (largest volume). Controls were the untreated cells, TSP-1 treatment, and DMSO treatment. This is the result of two independent experiments with similar in results for **A** and **B**. For **C** and **D** are the result of two independent experiments.

The effect of TSP-1 with or without Src family kinase inhibitor on the lipolytic activity of normal prostate epithelial cells and PCa cells

In RWPE-1 cells, lipolytic activity was significantly decreased with PP2 inhibitor treatment alone as compared to the untreated group (Figure 12A; P-value <0.021). Likewise, 5 nM TSP-1 with PP2 inhibitor treatment decreased lipolytic activity compared to the 5 nM TSP-1 group (P-value <0.008). In addition, as shown in Figure 12A, the TSP-1 at 20 nM treatment with PP2 inhibitor significantly decreased lipolysis of RWPE-1 cells as compared to TSP-1, 20nM (P-value <0.018). In PC-3 cells, lipolytic activity was not altered with the 5 nM TSP-1 treatment alone (Figure 12B). TSP-1 at the 20 nM dose decreased lipolysis, but this was not significantly different as compared to the untreated group (Figure 12B). In addition, with PP2 inhibitor treatment alone, a significant decrease of lipolysis was observed as compared to the untreated group (P-value <0.021). Compared to the 20 nM TSP-1 treatment alone, the TSP-1 at 20 nM treatment with PP2 inhibitor treatment decreased lipolytic activity (P-value <0.029). However, the 5 nM TSP-1 with Src family kinase inhibitor treatment did not affect lipolysis levels in PC-3 cells.

In LNCaP cells, the 5 or 20 nM TSP-1 treatment alone decreased lipolysis; yet, these changes were not statistically significant (Figure 12C). With PP2 inhibitor treatment alone, an increase in lipolysis was observed in LNCaP cells.

Likewise, TSP-1 at the 5 or 20 nM in the presence of PP2 inhibitor treatment induced lipolysis (Figure 12C). No statistically significant difference was observed in this study as compared to related groups (Figure 12C). A second, independent experiment on LNCaP cells, however, showed contrary results. In this experiment, TSP-1 treatment at the 5 or 20 nM increased lipolytic activity, but this was not statistically different (Figure 12D). However, PP2 treatment significantly decreased lipolysis of LNCaP as shown in Figure 12D (P-value <0.001). As presented in Figure 12D, the 5 nM TSP-1 treatment with PP2 inhibitor treatment significantly decreased lipolytic activity in LNCaP cells as compared to the 5 nM TSP-1 group (P-value <0.001) and the Src inhibitor group (P-value <0.001). Similar data were obtained with the 20 nM TSP-1 with PP2 inhibitor treatment as compared to the TSP-1 at 20nM group (P-value <0.001) and the Src inhibitor group (P-value <0.001). The DMSO control significantly decreased lipolytic activity of LNCaP cells compared to untreated group (Figure 12D; P-value <0.001).

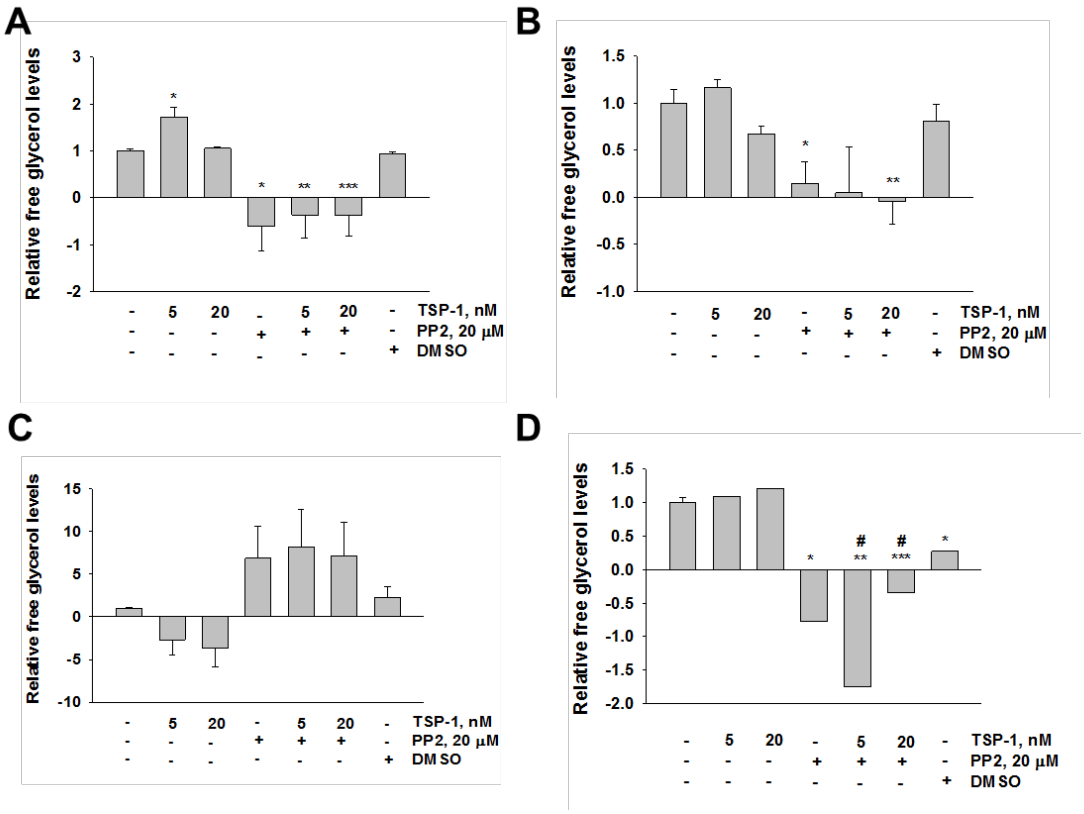

Figure 12. The effect of TSP-1 with chemical inhibitor of Src family kinase on lipolytic activity in RWPE-1, PC-3 and LNCaP cells. Both RWPE-1 **(A)** and PC-3 **(B)** were plated at 20,000 cells/cm2, and LNCaP cells **(C** and **D)** were plated at 40,000 cells/cm2 in tissue culture dishes. All cells were incubated overnight. Cells were then treated with TSP-1 with or without chemical inhibitor of Src family kinase, PP2, and incubated for 48 hours. Serum-free conditioned media was then collected and lipolytic activity quantified using the free glycerol assay. Data were normalized to total cell count and compared as fold over values. There were significant differences between groups. In (**A)**, *P-value <0.021 compared to untreated group; **P-value <0.008 compared to TSP-1, 5nM group; ***P-value <0.018 compared to TSP-1, 20nM group. In **(B)**, *P-value <0.021 compared to untreated group; **P-value <0.029 compared to TSP-1, 20nM. No statistical differences were found in **(C)** cells. In **(D**), *P-value <0.001 compared to untreated group; **P-value <0.001 compared to TSP-1, 5nM group; ***P-value <0.001 compared to TSP-1, 20nM group; #P-value <0.001 compared to JNK inhibitor group. DMSO control was an equivalent volume to PP2 dose (largest volume). Controls, which were the untreated cells, TSP-1 treatment, and DMSO treatment, were the same as shown in Figure 11. This is the result of two independent experiments with similar results for **A** and **B**. For **C** and **D** are the result of two independent experiments.

DISCUSSION

TSP-1 has been identified as a functional inhibitor of angiogenesis secreted by prostate epithelial cells [26]. The decreased level of TSP-1 in PCa, combined with the increased level of angiogenesis inducers, such as VEGF and fibroblast growth factor in PCa tissue [26], creates a pro-angiogenic environment. PEDF levels have also been shown to be decreased in PCa tissues [25]. In other tissue types, some studies have reported a link between TSP-1 and PEDF. For example, one study showed a positive association between expression levels of TSP-1 and PEDF in the vitreous fluid of diabetic patients [84]. Another study revealed that in pancreatic cells, PEDF overexpression inhibited TSP-1 levels [85]. The relationship between TSP-1 and PEDF in prostate tissues has not been well studied. A few recent studies in prostate cells; however, have shown that TSP-1 induces PEDF expression and lipolytic activity (Doll et al., unpublished data).

The molecular pathways through which TSP-1 signals in prostate cells is currently unexplored. In endothelial cells, where TSP-1 signaling has been well studied, the anti-angiogenic activity of TSP-1 is mediated through the intracellular signaling molecules, JNK and Fyn kinases [54]. JNK inhibition decreased endothelial cell proliferation and migration *in vitro* [65]. Furthermore, TSP-1 is unable to inhibit corneal angiogenesis in Fyn null mice or in wild type

mice in the presence of inhibitors that block the activity of p38 [66]. The question addressed in this study was whether or not TSP-1- induced PEDF expression and/or lipolytic activity was dependent on induction of JNK and/or Src family kinase signaling in RWPE-1 normal prostate epithelial cells and in the PCa cell lines, LNCaP and PC-3.

In this study, TSP-1 treatment did not alter the proliferation or viability of RWPE-1 cells. In a previous study, neither TSP-1 treatment at 5 nM nor 20 nM changed the proliferation or viability of RWPE-1 cells (Doll et al., unpublished data). RWPE-1 cells did not show significant changes in proliferation rate or in viability when exposed to the JNK inhibitor treatment, alone or with co-treatment with TSP-1. Similarly, proliferation of RWPE-1 was not altered with the Src family kinase inhibitor alone or with TSP-1. However, viability was reduced with with the Src family kinase inhibitor alone or with the Src family kinase inhibitor with the 20 nM TSP-1 treatment. Together, these data support that TSP-1, JNK, or Src family kinase signaling pathways are not required for RWPE-1 proliferation.

PEDF is down-regulated in PCa, where its loss has been linked to a metastatic phenotype [30]. In human malignant U251 glioma cells, an increase in PEDF expression stimulates TSP-1 expression [61]. Another study showed that PEDF expression increased TSP-1 levels in KM12 colon cancer cells, which led to decreased microvessel density [62]. In the present study, secreted PEDF levels in

RWPE-1 cells were not significantly altered with TSP-1 treatment alone. In two previous experiments in the our lab, inconsistent results were obtained. The first experiment showed that PEDF levels were significantly increased with 5 or 20 nM TSP-1 (Doll et al., unpublished data); however, in the second experiment, secreted PEDF levels were not affected by 5 or 20 nM TSP-1 treatment, and my data is consistent with this second observation. One likely explanation for the differences that I observed in response to TSP-1 treatment between these experiments is that the difference lots of TSP-1 were used. It is well known that TSP-1 is a large, 450-kDa glycoprotein, having several domains interacting with numerous cell receptors and proteases involved in angiogenesis [86]. TSP-1 can have different activity through protein interactions. However, when a specific domain of TSP-1 is cleaved, the remaining anti-angiogenic domains have been shown to have decreased anti-angiogenic activity [87]. Thus, the lot of TSP-1 and/or TSP-1 degradation could have been a factor.

Interestingly, secreted PEDF levels were reduced with JNK inhibition alone in RWPE-1 cells. This suggests that JNK signaling plays a significant role in the regulation of PEDF expression in RWPE-1 cells. The TSP-1 treatment modestly suppressed the JNK-inhibitor-mediated decrease in PEDF levels, but these levels were still significantly decreased compared to TSP-1 treatment alone. Thus, both TSP-1 and JNK molecule seem to function in the regulation of secreted PEDF levels in RWPE-1 cells. However, these results have to be viewed with some

caution, as the DMSO control showed an increase in PEDF levels in CM of RWPE-1 cells, and this increase was significantly different compared to untreated cells. Consequently, it is necessary to examine if DMSO treatment affects PEDF levels in RWPE-1 cells. Data obtained on secreted PEDF of RWPE-1 cells produced somewhat differing results with PP2 treatment alone or with TSP-1 at 5 or 20 nM treatment in two independent experiments. Further investigations are necessary to confirm these effects.

PEDF has been intensively studied as a secreted protein, while more recently, it has been studied for its intracellular functions. Intracellular PEDF has been detected in both the cytoplasm and nucleus in several cell types, including retinal pigment epithelial, retinoblastoma, neuroblastoma, and hepatocarcinoma cells [88, 89]. In these experiments, intracellular PEDF in RWPE-1 cells was not significantly changed with TSP-1 treatment. In previous studies, two independent experiments showed different results. In the first of these, 5 or 20 nM TSP-1 treatment did not alter PEDF expression in RWPE-1 CL (Doll et al., unpublished data). However, in the second previous experiment, treatment with 5 or 20 nM TSP-1 decreased PEDF levels compared to untreated cells (Doll et al., unpublished data). My data were consistent with the first experiment; however, the experiment should be repeated at least one more time to confirm these results.

JNK inhibition alone or in combination with TSP-1 at 20 nM did not altere intracellular PEDF levels in RWPE-1 cells, while TSP-1 at 5 nM with JNK inhibition significantly decreased PEDF. The DMSO control decreased PEDF expression, but no significant differences were observed between the DMSO control and the TSP-1 at 5 nM with JNK inhibitor treatment; therefore, TSP-1 could regulate PEDF expression through JNK pathway in RWPE-1. With Src family kinase inhibition or in combination with TSP-1 treatment, no change in expression levels of PEDF was observed in the RWPE-1 CL. These observations suggested that Src family kinases do not play a role in the regulation of intracellular PEDF expression in RWPE-1 cells.

Dysregulation in lipid metabolism is an established hallmark of cancer. Dai et al. showed that intracellular PEDF interacts with and facilitates ATGL translocation onto cellular lipid droplets, which then induces triglyceride degradation in hepatocytes [90]. In addition, knockdown of endogenous PEDF in liver cells decreased triglyceride degradation [90]. It has been proposed that PEDF also plays a role in lipid metabolism in PCa (Doll et al., unpublished data). In the present study, data obtained from RWPE-1 cells treated with 5 nM TSP-1 showed an increase in lipolysis. These data were consistent with the previous work (Doll et al., unpublished data). JNK inhibition alone or JNK inhibition with the 5 or 20 nM TSP-1 treatment significantly reduced lipolytic activity in RWPE-1 cells. Likewise, a reduction in lipolytic activity was identified with inhibition of the

Src family kinases, alone or in combination with TSP-1. These results indicated that TSP-1- induced lipolytic activity is dependent on JNK activation and/or Src family kinase activation in RWPE-1.

In this study, neither the 5 nor the 20 nM TSP-1 treatment affected PC-3 cell proliferation. However, in a previous study, the TSP-1 at the 5 nM treatment increased proliferation of PC-3 cells (Doll et al., unpublished data); therefore, a repeat experiment should be performed to clarify this discrepancy. The viability of PC-3 cells was not altered with TSP-1 treatment in the present study, which is consistent with the previous study in the lab (Doll et al., unpublished data). PC-3 cell proliferation was not altered with JNK inhibition alone or in combination with TSP-1. Interestingly, the 5 nM TSP-1 with JNK inhibition treatment slightly increased the viability of PC-3 cells, which suggests that TSP-1 treatment in the presence of JNK inhibition plays a role in PC-3 cell viability. Neither the Src family kinase inhibition alone nor in combination with TSP-1 altered the proliferation or the viability of PC-3 cells.

With respect to lipolytic activity, while TSP-1 treatment at the 5 nM dose slightly increased lipolysis in PC-3 cells, decreased activity was observed with the 20 nM TSP-1 dose. However, neither change was statistically significant compared to untreated cells. In a previous study, 20 nM TSP-1 treatment significantly increased lipolytic activity, whereas 5 nM TSP-1 slightly decreased

lipolytic activity in the PC-3 cells (Doll et al., unpublished data). This inconsistent data between studies may be due to differences in the activity of TSP-1 between lots of protein, as discussed above. These findings suggest that future studies should analyze the effect of TSP-1 on lipolytic activity in PC-3 to determine if a true difference exists. The JNK inhibitor treatment alone or in combination with TSP-1 treatment decreased lipolytic activity of PC-3 cells. Inhibition of the Src family kinases alone or in combination with 5 or 20 nM TSP-1 treatment also resulted in a reduction in lipolytic activity. Since these results are from two independent experiments, these studies would support that lipolytic activity in PC-3 cells is, in part, regulated by JNK and/or Src family kinase activity.

LNCaP proliferation was not altered with TSP-1 treatment; however, in a previous study, TSP-1 treatment increased LNCaP proliferation (Doll et al., unpublished data). This inconsistency could also be due to the fact that different lots of TSP-1 protein were used between my and the previous study. In LNCaP cells, there was no statistically significant decrease of viability observed with TSP-1 treatment. This observation is consistent with the prior investigation (Doll et al., unpublished data). The JNK inhibition alone or with TSP-1 treatment had no effect on LNCaP cell proliferation. In addition, TSP-1 at 20 nM with JNK inhibition decreased viability in LNCaP cells. The Src family kinase inhibition alone or with TSP-1 treatment did not alter LNCaP cell proliferation. In contrast, a reduction in LNCaP cell viability was observed with the Src family kinase

inhibition alone or in combination with 5 nM TSP-1, but these results were not statistically significant. However, a statistically significant decrease in LNCaP viability was observed with 5 nM TSP-1 with Src family kinase inhibition. A study reported that inhibition of Src family kinase activation decreased LNCaP growth [91]. In addition, in pancreatic cancer cells, proliferation was suppressed with Src inhibitors [92]. My data are consistent with these studies. Thus, these data suggest that Src family kinases play a role in the viability in LNCaP cells and that may modify its effect.

TSP-1 at 5 nM treatment increased secreted PEDF levels, while the 20 nM TSP-1 treatment decreased PEDF levels, in LNCaP cells. However, neither dose was statistically significant compared to untreated cells. The increased PEDF with 5 nM TSP-1 treatment is consistent with a previous study in the lab (Doll et al., unpublished data); however, the 20 nM TSP-1 treatment was inconsistent with previous data, which showed an increase in secreted PEDF levels. As previously stated, the differences in results could be due to a degradation of TSP-1 and/or difference in lot activity. Based on the variation of PEDF levels observed in these two studies, further experiments are necessary to clarify if TSP-1 does induce PEDF expression in LNCaP cells.

JNK inhibition decreased secreted PEDF protein levels from LNCaP cells, but this result was not statistically significant. However, TSP-1 at 5 nM with JNK

inhibition significantly reduced secreted PEDF. These findings suggest that JNK play a role in the regulation of secreted PEDF in LNCaP cells and that TSP-1 may modify its effect. Surprisingly, the Src family kinase inhibition alone or in combination with 5 or 20 nM TSP-1 elevated secreted PEDF levels. These results indicated that the Src family kinases play an important role in suppressing secreted PEDF levels. Together, the mechanism linking PEDF expression and JNK or Src family kianse remains unclear in LNCaP cells; therefore, further investigations are required to confirm the effects of TSP-1 with JNK or Src family kinase inhibition on secreted PEDF expression in LNCaP cells.

In LNCaP CL collected from cells treated with 5 nM TSP-1, a small increase in PEDF levels was observed, while with 20 nM TSP-1 treatment, there was a slight decrease in PEDF expression. A previous study in our lab showed that both doses of TSP-1 (5 or 20 nM) increased intracellular PEDF levels; thus, my data at the 20 nM dose is inconsistent with this previous study. The differences in the results between my data and the previous data could be due to several factors. In the prior study, the LNCaP cells were plated at 20,000 cells/cm^2, whereas I plated the LNCaP cells at 40,000 cells/cm^2. Therefore, the cell density might affect how the cells respond to TSP-1 doses as well as the lots and activity levels of TSP-1 used.

In LNCaP cells, the JNK inhibition treatment alone or with TSP-1 at 20 nM dose increased intracellular PEDF protein levels; however, no statistical differences were observed among groups. PEDF levels were significantly increased with 5 nM TSP-1 treatment in the presence of the JNK inhibitor treatment. This result indicates that the JNK pathway did not mediated the TSP-1-induced intracellular PEDF in LNCaP cells. The Src family kinase inhibition treatment, alone or with TSP-1, increased intracellular PEDF levels in LNCaP cells. In reported study, the authors showed that LNCaP cells overexpressed Src family kinases [93]. In the present study, inhibition of Src family kinase activity elevated both secreted and intracellular PEDF levels in LNCaP cells. Together, these data suggest that increased Src activity in LNCaP cells down-regulates PEDF expression. To determine if Fyn specifically, or other Src family kinase members, mediates TSP-1 induced intra- or extracellular PEDF expression in LNCaP cells, further investigations using a specific inhibitor, if available, or siRNA technologies, are necessary.

Lipolytic activity was measured in three independent studies of LNCaP cells, and conflicting results were obtained in each experiment. One of these experiments was consistent with a previous study, which showed an increase in lipolytic activity with TSP-1 treatment. The JNK inhibition treatment, alone or in combination with TSP-1 treatment, showed inconsistent effect on lipolytic activity in LNCaP cells. Likewise, conflicting data were also observed with Src family

kinase inhibition alone or with TSP-1 treatment. Even though these data were inconsistent, it seems possible that TSP-1, JNK and/or Src kinase signaling play a significant role in the regulation of lipolysis in LNCaP cells. However, further studies are required before firm conclusions can be made.

While some studies report that an absence of TSP-1 expression is correlated with a poor prognosis in PCa, in this study, TSP-1 did not inhibit PCa cell proliferation *in vitro* [94]. Similar to my data, a study proposed that there was no correlation between TSP-1 expression and cancer cell proliferation in renal cell carcinoma tissues [95]. Likewise, cell proliferation in breast cancer cells was not altered with TSP-1 expression [96]. However, a study reported that TSP-1 expression suppressed proliferation of small cell lung carcinoma [97]. Therefore, the importance of TSP-1 seems to vary and shows different effects on different types of cells or tissues.

I observed that inhibition of JNK activity led to reduced extracellular PEDF levels in RWPE-1 and LNCaP cells. JNK deficiency reduced tumor development in prostate [98], lymphoma [99], and lung cancer models [100]. I found that JNK activation suppressed expression of PEDF, a potent tumor suppressor in PCa. In the present study, JNK and/or Src family kinase inhibition alone or with TSP-1 inhibited lipolytic activity in RWPE-1 and PC-3 cells. Consistent with these data, it has been reported that the JNK pathway could play a role in the regulation of

lipolysis in mouse adipocytes [101]. Another group investigated Src activity in mouse 3T3-L1 preadipocytes and found that blocking Src family kinases, using either PP2 or siRNA inhibited lipolysis [102].

One explanation for the differences in response to treatment that I observed in my study between cell lines is that the genetic background of the cells. While PC-3 is an androgen-insensitive cell line, LNCaP cells are androgen-sensitive. In addition, PC-3 is derived from bone marrow whereas LNCaP cells are derived from lymph node [81, 82]. For example, PTEN gene plays a role in cellular senescence, and the three cell lines show different patterns of expression in this gene. A study found that JNK activity was increased in PTEN null cells [103]. Therefore, differential response of PC-3 and LNCaP cells to TSP-1 with either JNK or Src family kinase inhibitor treatments may be due to some defects in the signaling pathways.

CONCLUSION

To my knowledge, this is the first study investigating if TSP-1 activity is regulated via either JNK or Src family kinase signaling pathways. My data clearly demonstrates that TSP-1, JNK and/or Src family kinases could regulate anti-tumor activity in PCa cells. The central hypothesis of this study was that TSP-1-induced PEDF expression and/or lipolytic activity was dependent on induction of JNK and/or Fyn signaling in RWPE-1 normal prostate epithelial cells and in the PCa cell lines, LNCaP and PC-3. I investigated proliferation, viability, PEDF levels, and lipolytic activity *in vitro*. Viability was slightly altered with the TSP-1 treatment with the JNK inhibitor treatment or the Src family kinase inhibitor treatment in RWEP-1, PC-3 and LNCaP cells. In RWPE-1 and LNCaP cells, secreted PEDF levels were decreased with the JNK inhabitation, alone or with TSP-1 treatment. However, TSP-1 treatment in the presence of the Src family kinase inhibition increased secreted PEDF levels in LNCaP cells. Similarly, intracellular PEDF expression was elevated by TSP-1 with PP2 inhibitor treatment in LNCaP cells. Lipolytic activity was significantly suppressed by TSP-1 treatment with JNK or Src family kinase inhibition in RWPE-1 and PC-3. In this study, TSP-1 did not induce PEDF expression and/or lipolytic activity in PCa cells. However, I found that the activation of JNK and/or Src family kinases might be a significant regulator in induction of PEDF expression in RWEP-1 and LNCaP cells. In

addition, the activity of JNK or Src family kinases could regulate lipolytic activity in RWPE-1 and PC-3. Since Src family kinases are composed of several members [104], and PP2 is not a Fyn-specific inhibitor, future experiments, using an siRNA approach would be necessary to specifically test if Fyn, or other Src family kinase members, are required for this activity.

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