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## Differential Tumorigenic Potential and Matriptase Activation between PDGF B versus PDGF D in Prostate Cancer

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### Abstract

The platelet-derived growth factors (PDGF A, B, C, and D) and their receptors ( $\alpha$ -PDGFR and  $\beta$ -PDGFR) play an indispensable role in physiologic and pathologic conditions, including tumorigenesis. The transformative  $\beta$ -PDGFR is overexpressed and activated during prostate cancer progression, but the identification and functional significance of its complementary ligand have not been elucidated. This study examined potential oncogenic functions of  $\beta$ -PDGFR ligands PDGF B and PDGF D, using nonmalignant prostate epithelial cells engineered to overexpress these ligands. In our models, PDGF D induced cell migration and invasion more effectively than PDGF B *in vitro*. Importantly, PDGF D supported prostate epithelial cell tumorigenesis *in vivo* and showed increased tumor angiogenesis compared with PDGF B. Autocrine signaling analysis of the mitogen-activated protein kinase and phosphoinositide 3-kinase pathways found PDGF D-specific activation of the *c-jun*-NH<sub>2</sub>-kinase (JNK) signaling cascade. Using short hairpin RNA and pharmacologic inhibitors, we showed that PDGFD-mediated phenotypic transformation is  $\beta$ -PDGFR and JNK dependent. Importantly, we made a novel finding of PDGF D-specific increase in the shedding and activation of the serine protease matriptase in prostate epithelial cells. Our study, for the first time to our knowledge, showed ligand-specific  $\beta$ -PDGFR signaling as well as PDGF D-specific regulation of matriptase activity and its spatial distribution through shedding. Taken together with our previous finding that matriptase is a proteolytic activator of PDGF D, this study provides a molecular insight into signal amplification of the proteolytic network and PDGF signaling loop during cancer progression.

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No potential conflicts of interest were disclosed.

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## Introduction

The platelet-derived growth factor receptors ( $\alpha$ -PDGFR and  $\beta$ -PDGFR) play critical roles in lung, heart, central nervous system, and kidney development (1, 2). In addition to their function in normal physiologic processes, the PDGFR family is involved in many diseases such as atherosclerosis, glomerulonephritis, and cancer (3–5). PDGFRs are activated by the complementary ligands encoded by 4 distinct genes (PDGF A, B, C, and D) producing a combination of 5 homo- or heterodimeric growth factors (PDGF AA, BB, AB, CC, and DD; ref. 6). Whereas the classic PDGF ligands, PDGF A and PDGF B, are secreted as active dimers, the newly characterized PDGF C and PDGF D are secreted as latent growth factors that require proteolytic removal of the inhibitory N-terminal CUB domain by extracellular serine proteases to generate biologically active growth factor domain dimers (7–9). These dimeric polypeptides bind onto their cognate receptors at a 1:1 ratio with  $\alpha$ -PDGFR binding to PDGF AA, AB, and CC;  $\alpha\beta$ -PDGFR ligating with PDGF AB, BB, CC, and DD; and  $\beta\beta$ -PDGFR activated by PDGF BB and DD (3, 10). Receptor activation can mediate many cellular processes such as cell proliferation, migration, survival, transformation, and differentiation through the activation of downstream messengers including the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) pathway (11). PDGF signaling is critical for cell–cell communication as PDGF ligands are expressed and secreted by epithelial or endothelial cells to recruit and activate PDGF receptors expressed in stromal components such as smooth muscle cells, pericytes, and fibroblasts (1). During cancer progression, cancer cells express both PDGF ligand and its cognate receptors, thereby inducing both autocrine and paracrine signal transduction pathways (12, 13). Studies have reported variations in the expression levels and differential roles between  $\alpha$ -PDGFR and  $\beta$ -PDGFR depending on the tumor type (2, 7). However, little is known about PDGF ligand–specific PDGFR signaling and subsequent functional effects on tumor development and progression.

In prostate cancer, the most diagnosed cancer and the second leading cause of cancer-related death in American men,  $\beta$ -PDGFR is frequently upregulated and activated (14, 15). In fact, this receptor is overexpressed in 88% of primary prostate cancer and 80% of metastatic bone lesions (16). Furthermore,  $\beta$ -PDGFR is reported to be a part of a 5-gene signature predicting the course of prostate cancer progression following radical prostatectomy (17). Our recent study identified PDGF D as a ligand for  $\beta$ -PDGFR and the serine protease matriptase as its extracellular proteolytic activator in prostate cancer, suggesting a signaling axis of matriptase/PDGF D/ $\beta$ -PDGFR during prostate cancer progression (9). Interestingly, loss of the tumor suppressor phosphatase and tensin homologue deleted on chromosome 10 (PTEN) results in the upregulation of PDGF D and  $\beta$ -PDGFR expression, accompanied by decreased PDGF B expression in prostate epithelial cells (18). At present, it is unclear whether the PDGF ligand switch for  $\beta$ -PDGFR is a mere reflection of temporal and spatial regulation of PDGF ligands during physiologic and pathologic conditions or whether PDGF D activation of  $\beta$ -PDGFR functionally differs from PDGF B activation of  $\beta$ -PDGFR.

In this study, we addressed the question of whether  $\beta$ -PDGFR mediates unique intracellular signal transduction pathways in a ligand-specific manner, resulting in differential oncogenic effects. Here, we show that PDGF D is a more potent signaling molecule than PDGF B for the induction of prostate epithelial cell migration, invasion, and tumorigenesis. This transforming potential is dependent on PDGF D–specific activation of  $\beta$ -PDGFR/JNK signaling axis. In addition,  $\beta$ -PDGFR activation results in shedding and activation of matriptase in a PDGF D–specific manner, suggesting signal amplification of the matriptase/PDGF D/ $\beta$ -PDGFR functional loop. Considering that matriptase is frequently upregulated in multiple cancers, including ovarian, breast, lung, and prostate cancer (19), our novel discoveries have potentially broad biologic implications in human cancers.

## Materials and Methods

### Cell culture

Murine prostate epithelial cells (mPrEC; refs. 20, 21), a gift from Dr. Yong Chen at Wake Forest University School of Medicine, were maintained in Advanced Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% FBS (Invitrogen). Human benign prostatic hyperplasia BPH-1 cells (22), a gift from Dr. Simon Hayward at Vanderbilt University, were grown in 10% FBS RPMI, whereas NIH3T3 cells (American Type Culture Collection) were maintained in 10% FBS DMEM/F12. All growth media were supplemented with 2 mmol/L glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin (Invitrogen).

### Reagents

Anti-PDGF B antibody was from Millipore, whereas the anti-PDGF D antibody was custom made as described in Ustach and colleagues (23). Antiphospho- and total  $\beta$ -PDGFR, Erk, c-jun-NH<sub>2</sub>-kinase (JNK), p38, and Akt plus phospho-MKK4, c-Jun, and ATF-2 were from Cell Signaling Technology. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was from Santa Cruz Biotechnology. Active (M69) and total matriptase (M32) antibodies were a gift from Dr. Chen-Yong Lin at the University of Maryland. Anti-HAI-1 (human and murine) was from R&D Systems. Anti-CD31 antibody was from AbCam. JNK inhibitor SP600125 was from Caymen chemical.

### Overexpression of PDGF B and PDGF D in prostate epithelial cells

Human PDGF B cDNA was obtained from Open Biosystems (Catalog # MHS1010-7507640) and subcloned into *Kpn I* and *Xba I* sites within pcDNA3.1-Hygromycin vector and confirmed by DNA sequence analysis. Full-length human PDGF D cloning into pcDNA3.1-Neomycin was described in Ustach and colleagues (23). To subclone PDGF D into a Hygromycin vector, PDGF D containing pcDNA3.1 vector was digested with *Afl II* and *Xho I*, then ligated into pcDNA3.1-Hygromycin vector. Empty vector (Hygro or Neo), PDGF B-, or PDGF D expression vectors were transfected into mPrECs using a BioRad Gene Pulser (BioRad). For BPH-1 cell transfection, Lipofectamine 2000 (Invitrogen) was used. Transfected cells were selected either with 200  $\mu$ g/mL Geneticin (G418) or Hygromycin and the resulting pooled population used for experimentation and referred to as Hygro, Neo, PDGF B, PDGF D mPrEC, or BPH-1 cells, accordingly. PDGF B and PDGF D expression was confirmed through reverse transcriptase PCR (RT-PCR) and immunoblotting of conditioned media or whole-cell lysate (WCL).

### Immunoblot and RT-PCR analyses

mPrECs or BPH-1 cells were grown to full confluence in 100-mm tissue culture plates, then washed twice with warm PBS to remove serum. Cells were then cultured in 5-mL serum-free media for 48 hours. Conditioned media was collected and cells were washed with PBS then scraped. Collected conditioned media was concentrated 50-fold per manufacturer's recommendation using an Amicon centrifugal filter unit (Millipore). An amount of 30  $\mu$ L of concentrated conditioned media was used for immunoblot analysis.

Scraped cells were used for RNA or protein analysis by RT-PCR and immunoblotting, respectively. RNA was isolated using the RNeasy mini kit (Qiagen) and RT-PCR detection for PDGF family members using primers in Supplementary Table S1 at 30 PCR cycles, with each cycle consisting of denaturation at 94°C for 90 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 150 seconds.

For protein analysis (autocrine signaling and matriptase expression), cells were lysed in radioimmunoprecipitation assay lysis buffer (Millipore) containing 1 mmol/L phenyl-

methylsulfonylfluoride, 2 mmol/L sodium orthovanadate, 1 mmol/L sodium fluoride, and a complete protease inhibitor cocktail (Roche) for 30 minutes, then centrifuged for another 20 minutes. Lysate was quantified using the BCA protein assay kit (Pierce Biotechnology) and 30  $\mu$ g of whole-cell lysate was used for immunoblot analysis. Protein and RNA expression analyses were repeated at least 3 independent times.

### ***In vitro* cell migration and Matrigel invasion**

Cell migration was carried out as described previously (24). Briefly, prostate epithelial cells grown to confluence then abraded using a 10- $\mu$ L pipette tip. Cells were washed with PBS and incubated in serum-free media supplemented with 25  $\mu$ g/mL Mitomycin C. Photographs of wound channel were taken at the indicated time points and remaining cleared area was quantified using NIH ImageJ software. For Matrigel invasion,  $7.5 \times 10^4$  cells were plated into a Matrigel-coated transwell (BD Biosciences) and allowed to invade toward serum containing growth media for 24 or 16 hours for mPrECs or BPH-1 cells, respectively. Transwells were cleaned with a cotton tip, and cells on the bottom of the filter were stained with crystal violet and quantified using a Nikon TMS-F inverted microscope at 100 $\times$  magnification. Experiments were carried out in triplicates and repeated at least 3 independent times.

### **PDGF B- and PDGF D-mediated paracrine activation of $\beta$ -PDGFR**

mPrECs and BPH-1 conditioned media was collected as described above and used to treat NIH3T3 cells. Serum-free media (SFM) or SFM plus 25 ng/mL recombinant PDGF B (Calbiochem) treatments were used as negative and positive controls, respectively. Fifteen minutes later, NIH3T3 cells were lysed for  $\beta$ -PDGFR and downstream signaling activation assays. Activation assays were carried out in 3 separate experiments.

### **shRNA-mediated knockdown of $\beta$ -PDGFR and matriptase in prostate epithelial cells**

Scrambled short hairpin (shRNA) sequence (shScrm; catalog # RHs4346), shRNA against  $\beta$ -PDGFR (sh $\beta$ R; catalog # RHS4430-101067973), or matriptase (shMat; catalog # RHS4430-101168342) was obtained from Open Biosystems. PDGF D expressing mPrECs or BPH-1 cells were grown to subconfluence, then infected with shScrm, sh $\beta$ -PDGFR, or shMatriptase lentivirus at 3 MOI (multiplicity of infection) per manufacturer's protocol. Cells were selected with 4  $\mu$ g/mL of puromycin and the resulting pooled population was used for experimentation. Down-regulation of gene expression was assessed by RT-PCR and immunoblot analyses.

### ***In vivo* tumorigenesis of BPH-1 cells**

Vector, PDGF B or PDGF D BPH-1 cells at  $5 \times 10^6$  cells per 100  $\mu$ L of serum-free media were injected subcutaneously into both flanks of 5-week-old-male C.B.-17 severe combined immunodeficient (SCID) mice (Taconic Farms). Five mice (10 injections) per group were used per experiment and *in vivo* analysis repeated twice. Tumor volume was monitored for 2 weeks by external caliper measurements and calculated as  $V = (L^2 \times l)/2$ , in which  $L$  and  $l$  represent small and large tumor diameter. At the conclusion of the experiments, tumors were excised and treated with 4% paraformaldehyde overnight, then paraffin embedded for sectioning and immunohistochemistry. Microvessel density measurement was carried out on CD31-stained sections at 200 $\times$  magnification and quantified using the CellSens Dimension (Olympus) program. Quantitation was assessed on 3 representative tumor sections per group. At least 12 high-powered fields (HPF) were analyzed per section and average microvessel counts were obtained.

### ***In vitro* cell proliferation**

Vector, PDGF B or PDGF D BPH-1 cells were plated in 24-well plates at  $1 \times 10^4$  cells per well and grown overnight in normal growth media. Cells were then washed twice with warm media then fed with 500  $\mu$ L serum-free media. For NIH3T3 coculture,  $7.5 \times 10^4$  NIH3T3 cells were plated into an 8- $\mu$ m transwell and incubated with the BPH-1 cells at the bottom of the well for 24 and 48 hours. Cell proliferation was monitored via Trypan Blue exclusion assay and plotted as a proliferation rate, which is the fold difference in proliferation at each time point normalized to time 0 (before coculture).

### **Statistical analysis**

Statistical significance was determined using unpaired Student *t* test, and differences were considered significant when *P* value was less than 0.05.

## **Results**

### **PDGF D is a more potent inducer of prostate epithelial cell migration and invasion than PDGF B**

To examine PDGF ligand-specific  $\beta$ -PDGFR functions in prostate epithelial cells, we established murine and human prostate epithelial cell lines (mPrEC and BPH-1, respectively) engineered to express PDGF B or PDGF D (Fig. 1A and B). PDGF B chain that consists of 241 amino acid residues is dimerized and subsequently undergoes posttranslational modifications and intracellular proteolytic processing at both N-terminal and C-terminal ends, generating intracellular intermediate forms that often differ in their molecular weights among cell types (25–28). Immunoblot analysis in reducing condition detected a PDGF B monomer with a molecular weight of approximately 18 kDa in PDGF B mPrEC and approximately 23 kDa in PDGF B BPH-1 cells (Fig. 1A and B). Unlike PDGF B, full-length PDGF D is secreted as a latent dimer (10). As shown in Fig. 1A and B, the 50-kDa full-length PDGF D monomer is detected in both PDGF D–overexpressing mPrECs and BPH-1 cells. When we assessed the effects of PDGF B and PDGF D on prostate epithelial cell motility by an *in vitro* scratch migration assay, PDGF D expression was more effective than PDGF B in inducing cell migration in both cell models as shown by greater reduction of the abraded wound channel over time (Fig. 1C and D; Supplementary Fig. S1A and B). Similarly, a cell invasion assay through Matrigel also showed PDGF D is a stronger inducer of an invasive phenotype in prostate epithelial cells compared with PDGF B (Fig. 1E and F).

### **PDGF D–specific autocrine signaling activates the JNK pathway**

To identify the intracellular signaling network responsible for the differential cellular effects mediated by PDGF B versus PDGF D, we examined the activation status of MAPK members, which are well-known PDGFR downstream signaling molecules regulating cell motility (3, 10, 11). In mPrECs, PDGF D autocrine signaling resulted in enhanced Erk and JNK activation in comparison with PDGF B cells (Fig. 2A). Although both PDGF B and PDGF D induced Erk activation in BPH-1 cells, JNK activation was specific to PDGF D (Fig. 2B). It should be noted that the well-known motility inducer p38 was not constitutively activated by autocrine signaling of neither PDGF B nor PDGF D in prostate epithelial cells. Overall, PDGF D–specific activation of JNK was consistent within our overexpression models and therefore we dissected upstream and downstream effectors of this secondary messenger. As shown in Fig. 2C and D, increased activation of the upstream JNK activator MKK4 as well as downstream targets of JNK, c-Jun, and ATF-2 were readily detected in PDGF D–expressing mPrE and BPH-1 cells (Fig. 2C and D). We observed a slight molecular weight shift of phosphorylated ATF-2 in our BPH-1 cells, which could be due to the sequential phosphorylation at Thr69, then Thr71 leading to its activation (29). To



ascertain that the failure of PDGF B to activate the JNK pathway in prostate epithelial cells is not due to insufficient production of biologically active PDGF B, we carried out paracrine stimulation of NIH3T3 cells with conditioned media derived from mPrEC and BPH-1 cells expressing PDGF B or PDGFD. PDGF B-containing conditioned media activated  $\beta$ -PDGFR and its downstream signaling molecule Akt more effectively than PDGF D-containing conditioned media (Supplementary Fig. S2A and B). These results showed that PDGF B production was, at least, comparable with PDGF D in our model systems and their differential autocrine effects are likely because of their intrinsic differences in their signaling capacity.

Although PDGF B can bind and activate both  $\alpha$ -PDGFRs and  $\beta$ -PDGFRs, PDGF D is specific to  $\beta$ -PDGFR. However, PDGF D dimer was suggested to activate  $\alpha\beta$ -PDGFR heterodimer in addition to  $\beta\beta$ -PDGFR homodimer (10, 30). Profiling of our prostate epithelial cells show both models mainly express  $\beta$ -PDGFR (Supplementary Fig. S3). To ascertain the functional significance of  $\beta$ -PDGFR for the mediation of PDGF D-specific signaling within prostate epithelial cells, we downregulated  $\beta$ -PDGFR in PDGF D-overexpressing mPrEC and BPH-1 cells using an shRNA approach. It should be noted that we were unable to detect  $\beta$ -PDGFR at the protein level in these cells, likely because of constitutive autocrine stimulation, which results in internalization and degradation of  $\beta$ -PDGFR. However, RT-PCR analysis exhibited effective knockdown of  $\beta$ -PDGFR at the RNA level (Fig. 2E). Moreover, shRNA-mediated  $\beta$ -PDGFR downregulation reduced the levels of active Erk, a typical downstream signal mediator of  $\beta$ -PDGFR, indirectly supporting shRNA-mediated  $\beta$ -PDGFR knockdown. Importantly, the level of active JNK was also reduced in PDGF D-expressing prostate epithelial cells in response to  $\beta$ -PDGFR knockdown (Fig. 2E), showing an indispensable role for  $\beta$ -PDGFR in PDGF D-specific signal transduction in prostate epithelial cells.

### **PDGF D/ $\beta$ -PDGFR/JNK signaling axis promotes a migratory/invasive phenotype of prostate epithelial cells**

Next, we investigated the role  $\beta$ -PDGFR plays in supporting PDGF D-mediated cells migration and invasion. Downregulation of  $\beta$ -PDGFR in mPrEC or BPH-1 cells significantly attenuated the migratory potential of prostate epithelial cells as evident in their inability to close the abraded wound channel (Fig. 3A and B and Supplementary Fig. S4A and B). Furthermore,  $\beta$ -PDGFR knockdown reduced Matrigel cell invasion in both PDGF D-expressing mPrEC and BPH-1 cells (Fig. 3C and D). To further characterize the involvement of the JNK pathway, we used a pharmacologic JNK inhibitor (Fig. 4A) and monitored PDGF D-specific cell migration and invasion. JNK inhibition effectively retarded wound channel closure of PDGF D mPrEC and BPH-1 cells (Fig. 4B and C; Supplementary Fig. S5A and B). In addition, JNK inhibition significantly abrogated PDGF D-induced Matrigel invasion (Fig. 4D and E).

### **PDGF D promotes matriptase shedding**

Previously, we identified matriptase as a PDGF D-inducible serine protease that can process the latent PDGF D dimer into an active growth domain dimer, suggesting a positive feedback loop between matriptase and PDGF D (9). In our current study, we aimed to assess whether matriptase modulation was specific to PDGF D or whether it is inclusive of PDGF B as well. Neither PDGF D nor PDGF B autocrine signaling significantly altered the matriptase mRNA levels in both mPrEC and BPH cell models (data not shown). Matriptase is a transmembrane serine protease and its active form is often detected as a complex with HAI-1 on the cell surface or shed into the extracellular milieu. Previous studies reported that, depending on proteolysis, the endogenous inhibitor of matriptase HAI-1 is detected as 50- and 40-kDa proteins in conditioned media or as a 55-kDa protein in cell lysates (31).

Here, we examined whether PDGF signaling regulates the levels and/or activation status of matriptase in prostate epithelial cells. As shown in Fig. 5A, antibody specific to activated matriptase detected higher levels of shed matriptase in the conditioned media collected from PDGF D–overexpressing BPH-1 cells compared with those from control or PDGF B–expressing cells (Fig. 5A). Although the total matriptase levels were comparable among cell lysates of vector (Hygro), PDGF B- and PDGF D–overexpressing BPH-1 cells, the matriptase level was higher in conditioned media from PDGF D BPH-1 cells compared with control and PDGF B BPH-1 cells. In addition, HAI-1 level was lower in PDGF D BPH-1 cell lysates, potentially because of shedding of the matriptase-HAI-1 complex into the extracellular milieu (Fig. 5A). These findings were corroborated in our mPrEC model, in which higher levels of shed matriptase were detected in conditioned media from PDGF D–overexpressing cells (Supplementary Fig. S6). Due to the fact that our antibody against active matriptase recognizes human matriptase only, we used a murine-specific HAI-1 antibody to detect activated matriptase-HAI-1 complex as a surrogate marker. We observed no difference in expression levels of free HAI-1 among control, PDGF B and PDGF D mPrECs. However, high molecular mass protein complexes, indicative of HAI-1/active matriptase complexes, were detected in PDGF D mPrECs (Supplementary Fig. S6). These results suggested that PDGF D signaling regulates matriptase posttranslationally, at the level of activation/shedding. Next, to test whether PDGF D regulates matriptase activation and shedding via signaling through  $\beta$ -PDGFR, we evaluated matriptase expression profile in PDGF D BPH-1 upon shRNA-mediated  $\beta$ -PDGFR knockdown. As shown in Fig. 5B, matriptase shedding was drastically attenuated in response to  $\beta$ -PDGFR downregulation, accompanied with increased HAI-1 in cell lysates. These results suggested the functional significance of PDGF D/ $\beta$ -PDGFR signaling axis for the regulation of matriptase at the levels of activation and shedding, possibly as a complex with HAI-1 in prostate epithelial cells.

### **Matriptase is essential for PDGF D–induced migratory/invasive phenotype**

The epithelial specific serine protease matriptase has been shown to mediate cancer cell migration and invasion, and it is associated with breast, prostate, and ovarian cancer progression (19, 32). To investigate the role matriptase plays in PDGF-associated phenotypic transformation, we downregulated its expression in PDGF D BPH-1 cells. Stable shRNA transduction targeting matriptase effectively reduced matriptase expression levels as shown by attenuated levels of total matriptase, as well as shed protease into cell culture medium (Fig. 6A). Interestingly, reduction of matriptase levels led to an increase in the amount of cellular HAI-1 that supports the intricate balance between matriptase and HAI-1 for the regulation of their spatial distribution and activity. PDGF D–enhanced migratory phenotype was effectively reversed upon matriptase knockdown as shown by an *in vitro* scratch migration assay (Fig. 6B and Supplementary Fig. S7). In addition, shRNA-mediated matriptase knockdown effectively abrogated the invasive phenotype of PDGF D BPH-1 cells (Fig. 6C).

### **PDGF D supports prostate epithelial cell tumorigenesis *in vivo***

Next, we analyzed the tumorigenic potential of PDGF B versus PDGF D using an *in vivo* tumorigenesis model of BPH-1. To this end, vector (Hygro), PDGF B or PDGF D BPH-1 cells were injected subcutaneously into the flanks of male SCID mice and the tumor volume was monitored. Seven days posttumor implantation, both PDGF B and PDGF D developed detectable tumor nodules when compared with the vector control. However, as time progressed, vector and PDGF B tumors reached a plateau, whereas PDGF D tumors continued to grow significantly larger (Fig. 7A and B). Tumors in all injected groups were confirmed by histologic analysis. As shown in Fig. 7C, immunohistochemical analysis revealed that matriptase expression was specific to PDGF D–overexpressing tumors

corroborating our *in vitro* findings in Fig. 5. Because angiogenesis is critical for tumor growth and PDGF D is known to support angiogenesis through blood vessel maintenance (33), we examined whether increased tumorigenic potential of PDGF D is associated with its angiogenic potential in our tumorigenesis model. Microvessel quantitation by CD31 staining showed significantly higher microvessel count in PDGF D BPH-1 tumors compared with the control or PDGF B BPH tumors (Fig. 7D). Interestingly, it was noticed that blood vessel staining was observed mainly in the stroma in all tumor samples.

To investigate the molecular/cellular basis for PDGF D-enhanced BPH-1 tumor growth *in vivo*, we carried out a cell proliferation assay. Neither PDGF B nor PDGF D had noticeable effect on BPH-1 cell proliferation (Fig. 7E). However, when cell proliferation was examined in the presence of the murine fibroblast NIH3T3 cells on top of transwell, the proliferation rate greatly increased in PDGF D BPH-1 cells compared with the Hygro control and PDGF B BPH-1 cells. These results suggested that although PDGF D autocrine signaling has little effect on BPH-1 cell proliferation, prostate carcinoma-produced PDGF D induces PDGFR-mediated signaling in stromal cells, resulting in secretion of soluble factors which, in turn, induce prostate epithelial cell proliferation in a secondary paracrine manner. Considering that pro-HGF is among the best known substrates for matriptase (32, 34), our results may also support the possibility that matriptase, shed by PDGF D autocrine signaling in BPH-1 cells, proteolytically activates pro-HGF secreted by stroma cells which, in turn, activates c-Met signaling for BPH-1 cell growth *in vivo*. Taken together, this study suggests diverse oncogenic potentials of PDGF D involving both autocrine and paracrine signaling mechanisms for tumor interactions with surrounding stroma.

## Discussion

In this study, we assessed the transforming properties of the  $\beta$ -PDGFR ligands, PDGF B and PDGF D, by using nonmalignant prostate epithelial cell models engineered to overexpress these ligands. We found PDGF D to be more effective than PDGF B in supporting cell migration, invasion, and tumorigenesis through the  $\beta$ -PDGFR/JNK signaling axis and matriptase activation/shedding. These results, for the first time to our knowledge, show the functional significance of PDGF ligand-specific cell signaling in prostate cancer. At present, the molecular mechanisms underlying PDGF D-specific  $\beta$ -PDGFR signaling are unclear. Unlike prostate epithelial cells, which express lower levels of PDGFRs with  $\beta$ -PDGFR being predominant, both PDGF B and PDGF D are highly transforming in the murine fibroblast NIH3T3, which express high levels of both  $\alpha$ -PDGFRs and  $\beta$ -PDGFRs (35). Similar to prostate epithelial cells, we previously showed PDGF D-specific cell signaling leading to osteoclast activation and intraosseous prostate cancer growth (36). Interestingly, osteoclasts also express low levels of PDGFRs, with  $\beta$ -PDGFR being predominant (36). Thus, it is plausible to hypothesize that when  $\beta$ -PDGFR levels are high, both PDGF B and PDGF D readily induce  $\beta$ -PDGFR dimerization and activation, resulting in classic  $\beta$ -PDGFR signal transduction pathways. In contrast, in cells with low levels of  $\beta$ -PDGFR, PDGF D-activated  $\beta$ -PDGFR signaling differ from PDGF B/  $\beta$ -PDGFR signaling, possibly due to differences in their binding kinetics/affinity or the involvement of the CUB domain of PDGF D for the recruitment of unique signaling molecules to the  $\beta$ -PDGFR signaling complex on the cell surface. It is also possible that PDGF D-specific signaling is cell-type specific, not necessarily associated with the  $\beta$ -PDGFR level.

Analysis of the autocrine signaling events between PDGF B and PDGF D showed a critical role of the  $\beta$ -PDGFR/JNK axis in PDGF D-mediated phenotypic transformation. These findings are corroborated by previous reports showing PDGF D activation of the JNK pathway through  $\beta$ -PDGFR in liver cells (5). Considering  $\beta$ -PDGFR-associated Nck activation of MEK1 and MKK4 in human kidney cells (37), it would be of interest to



examine  $\beta$ -PDGFR recruitment of Nck for JNK activation in prostate epithelial cells. Signal transduction downstream of PDGFR regulates many cellular processes, undoubtedly in collaboration with predisposed signaling programs, depending on the genetic background. PDGF D effectively induces migratory and invasive phenotypes in both human BPH-1 and normal mPreCs via activation of the JNK pathway. Consistently, PDGF D-mediated *in vivo* tumorigenesis was observed in the BPH-1 model. These results suggest that PDGF may be a competent factor requiring other genetic hits to progress into a bona fide transformed state (38).

Previously, PDGF D was shown to induce pancreatic, gastric, brain, and renal tumorigenesis (12, 39–41). Using an *in vivo* tumorigenesis model of BPH-1, we show here the oncogenic potential of PDGF D in prostate cancer. Interestingly, we observed that PDGF D is a more potent inducer of *in vivo* tumor growth, whereas both PDGF B and PDGF D trigger tumor onset and initial tumor growth. When tumor vasculature was assessed, PDGF D tumors were significantly more vascularized than their PDGF B counterparts. This may result from higher angiogenic potential of PDGF D, which has been reported to upregulate angiogenic factors such as VEGF and angiopoietin-1 (39–41). Although it is unclear whether the angiogenic potential of PDGF D is a prerequisite for its *in vivo* tumorigenic effects, or increased tumor vasculature in PDGF D BPH-1 tumor was secondary to the increased tumor size, this study clearly indicates oncogenic functions of PDGF D via both autocrine and paracrine mechanisms.

PDGF D was shown to enhance CXCR4 and protease activity, which may indirectly contribute to tumorigenic effects of PDGF D (39, 42). One of the proteases PDGF D is known to modulate is the type II transmembrane serine protease matriptase (9). Matriptase is an epithelial specific protease, involved in terminal epidermal differentiation and epidermal barrier function (43), as well as in cell migration, invasion, and proliferation through the proteolytic activation of its substrates including uPA, HGF, and ECM components (32). Matriptase is tightly regulated by its endogenous inhibitor HAI-1 and disruption of the HAI-1/matriptase expression ratio is thought to result in disease progression including tumorigenesis (31, 44). In fact, matriptase is upregulated in multiple cancers including ovarian, breast, lung, and prostate cancer (19). In our previous report, we showed both PDGF D and matriptase expression was significantly correlated with prostate cancer progression (9). Using the BPH-1 model, this study examined whether PDGF B may as well play a role in matriptase modulation and found that matriptase shedding and activation is PDGF D specific. The mechanism by which the PDGF D/ $\beta$ -PDGFR axis regulates matriptase is still unknown. Previous reports studying HER 2 (ErbB 2) showed a significant role of the PI3K/Akt 2 pathway in matriptase activation (45). However, in our model there is little difference in PI3K/Akt pathway between PDGF B versus PDGF D and the JNK pathway seems to be a key player in PDGF D-specific signaling in prostate epithelial cells. Interestingly, inhibition of JNK did not affect matriptase shedding suggesting a yet uncharacterized PDGF D-specific pathway is involved (data not shown). Transcriptional regulation of matriptase by testosterone has also been shown in LNCaP cells (45). We initially identified matriptase as a PDGF D-inducible gene by microarray analysis (9). Although PDGF D signaling results in a 2- to 3-fold increase in matriptase mRNA and protein levels, immunohistochemical analysis of matriptase using antibodies against activated matriptase showed drastic increases in a human prostate carcinoma LNCaP model (data not shown). These results suggest PDGF D regulation of matriptase on multiple levels, including gene expression and activation steps. In BPH-1 and mPreEC models, PDGF D signaling did not show a detectable change in the matriptase mRNA levels. Importantly, this study indicates that PDGF D upregulates matriptase at the level of its activation and/or shedding. This novel finding provides important biologic insight into functional interplay between PDGF signaling and serine protease matriptase, known to be a critical proteolytic

enzyme in multiple human cancers. PDGF D–mediated matriptase shedding may also indicate a new role for matriptase in ECM in addition to its known functions on the cell membrane.

For many years  $\beta$ -PDGFR activation has been shown in prostate cancer; however, the identification of its complementary ligand and their functional significance were largely unknown. In this study, we showed PDGF D–specific signaling critical for phenotypic transformation in prostate epithelial cells, providing rationale for the design of cancer-specific PDGF inhibitors. This is of particular importance because prostate cancer clinical trials with receptor tyrosine kinase inhibitors, such as Gleevec, were unsuccessful (46). Importantly, our previous preclinical study with the VEGFR/PDGFR inhibitor cediranib showed promise in reducing PDGF D–positive prostate tumor bone lesions (47). Taken together, this study not only signifies PDGF D as a ligand for  $\beta$ -PDGFR activation in prostate cancer but also may provide the molecular basis for biomarker-guided patient stratification and the development of cancer-specific therapy.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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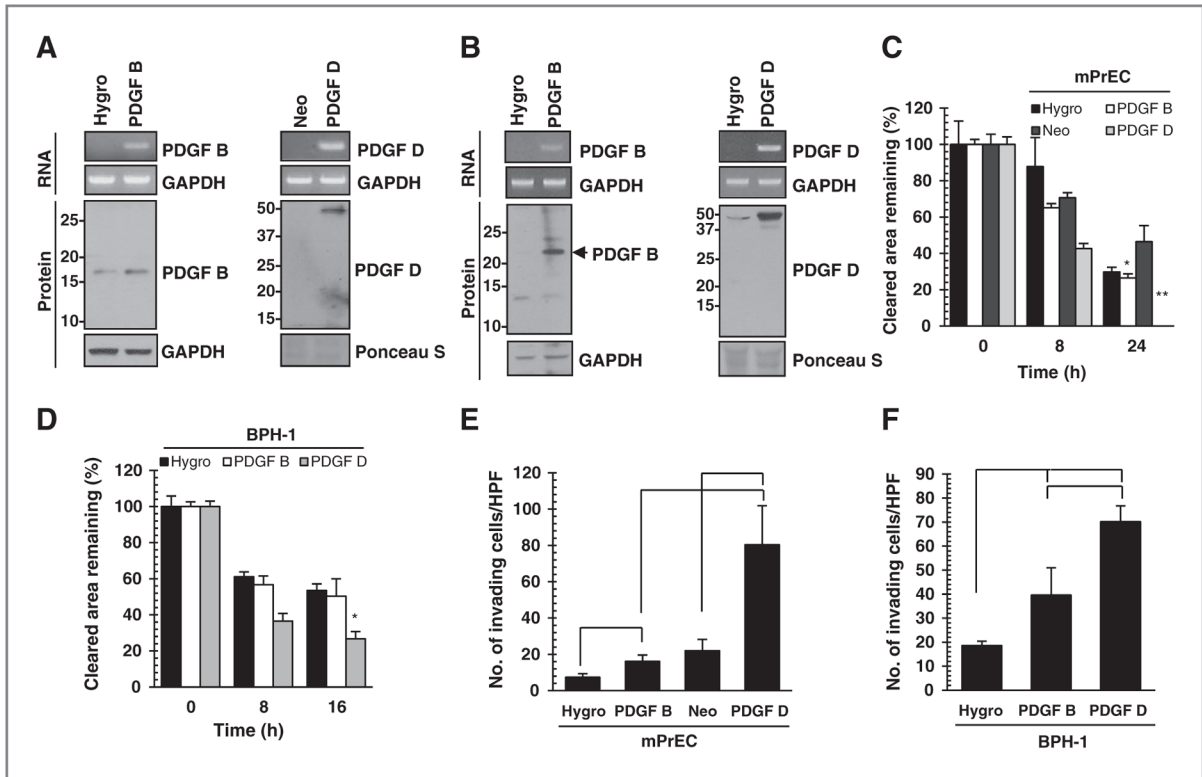
This work was supported by the NIH/National Cancer Institute grants RO1CA123362 and RO1CA064139 (to H-R. C. Kim), as well as the Ruth L. Kirschstein National Research Service Award F32-CA 142038 (to A.J. Najy).

## References

1. Betsholtz C, Karlsson L, Lindahl P. Developmental roles of platelet-derived growth factors. *Bioessays*. 2001; 23:494–507. [PubMed: 11385629]
2. Andrae J, Gallini R, Betsholtz C. Role of platelet-derived growth factors in physiology and medicine. *Genes Dev*. 2008; 22:1276–312. [PubMed: 18483217]
3. Heldin CH, Ostman A, Ronnstrand L. Signal transduction via platelet-derived growth factor receptors. *Biochim Biophys Acta*. 1998; 1378:F79–113. [PubMed: 9739761]
4. Karvinen H, Rutanen J, Leppanen O, Lach R, Levonen AL, Eriksson U, et al. PDGF-C and -D and their receptors PDGFR-alpha and PDGFR-beta in atherosclerotic human arteries. *Eur J Clin Invest*. 2009; 39:320–7. [PubMed: 19292888]
5. Borkham-Kamphorst E, van Roeyen CR, Ostendorf T, Floege J, Gressner AM, Weiskirchen R. Profibrogenic potential of PDGF-D in liver fibrosis. *J Hepatol*. 2007; 46:1064–74. [PubMed: 17397961]
6. Fredriksson L, Li H, Eriksson U. The PDGF family: four gene products form five dimeric isoforms. *Cytokine Growth Factor Rev*. 2004; 15:197–204. [PubMed: 15207811]
7. Reigstad LJ, Varhaug JE, Lillehaug JR. Structural and functional specificities of PDGF-C and PDGF-D, the novel members of the platelet-derived growth factors family. *FEBS J*. 2005; 272:5723–41. [PubMed: 16279938]
8. Hurst NJ Jr, Najy AJ, Ustach CV, Movilla L, Kim HR. Platelet-derived growth factor-C (PDGF-C) activation by serine proteases: implications for breast cancer progression. *Biochem J*. 2012; 441:909–18. [PubMed: 22035541]
9. Ustach CV, Huang W, Conley-LaComb MK, Lin CY, Che M, Abrams J, et al. A novel signaling axis of matriptase/PDGF-D/ss-PDGFR in human prostate cancer. *Cancer Res*. 2010; 70:9631–40. [PubMed: 21098708]

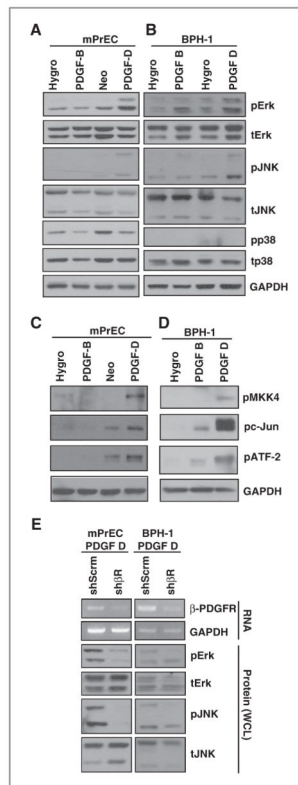
10. Yu J, Ustach C, Kim HR. Platelet-derived growth factor signaling and human cancer. *J Biochem Mol Biol.* 2003; 36:49–59. [PubMed: 12542975]
11. Wang Z, Ahmad A, Li Y, Kong D, Azmi AS, Banerjee S, et al. Emerging roles of PDGF-D signaling pathway in tumor development and progression. *Biochim Biophys Acta.* 2010; 1806:122–30. [PubMed: 20434526]
12. Lokker NA, Sullivan CM, Hollenbach SJ, Israel MA, Giese NA. Platelet-derived growth factor (PDGF) autocrine signaling regulates survival and mitogenic pathways in glioblastoma cells: evidence that the novel PDGF-C and PDGF-D ligands may play a role in the development of brain tumors. *Cancer Res.* 2002; 62:3729–35. [PubMed: 12097282]
13. Murata T, Mizushima H, Chinen I, Moribe H, Yagi S, Hoffman RM, et al. HB-EGF and PDGF mediate reciprocal interactions of carcinoma cells with cancer-associated fibroblasts to support progression of uterine cervical cancers. *Cancer Res.* 2011; 71:6633–42. [PubMed: 22009535]
14. Siegel R, Ward E, Brawley O, Jemal A. Cancer statistics, 2011: the impact of eliminating socioeconomic and racial disparities on premature cancer deaths. *CA Cancer J Clin.* 2011; 61:212–36. [PubMed: 21685461]
15. Uehara H, Kim SJ, Karashima T, Shepherd DL, Fan D, Tsan R, et al. Effects of blocking platelet-derived growth factor-receptor signaling in a mouse model of experimental prostate cancer bone metastases. *J Natl Cancer Inst.* 2003; 95:458–70. [PubMed: 12644539]
16. Ko YJ, Small EJ, Kabbinar F, Chachoua A, Taneja S, Reese D, et al. A multi-institutional phase ii study of SU101, a platelet-derived growth factor receptor inhibitor, for patients with hormone-refractory prostate cancer. *Clin Cancer Res.* 2001; 7:800–5. [PubMed: 11309325]
17. Singh D, Febbo PG, Ross K, Jackson DG, Manola J, Ladd C, et al. Gene expression correlates of clinical prostate cancer behavior. *Cancer cell.* 2002; 1:203–9. [PubMed: 12086878]
18. Conley-LaComb MK, Huang W, Wang S, Shi D, Jung YS, Najy A, et al. PTEN regulates PDGF ligand switch for beta-PDGFR signaling in prostate cancer. *Am J Pathol.* 2012; 180:1017–27. [PubMed: 22209699]
19. List K. Matriptase: a culprit in cancer? *Future Oncol (London, England).* 2009; 5:97–104.
20. Berquin IM, Min Y, Wu R, Wu J, Perry D, Cline JM, et al. Modulation of prostate cancer genetic risk by omega-3 and omega-6 fatty acids. *J Clin Invest.* 2007; 117:1866–75. [PubMed: 17607361]
21. Wang S, Wu J, Suburu J, Gu Z, Cai J, Axanova LS, et al. Effect of dietary polyunsaturated fatty acids on castration-resistant Pten-null prostate cancer. *Carcinogenesis.* 2012; 33:404–12. [PubMed: 22159221]
22. Hayward SW, Dahiya R, Cunha GR, Bartek J, Deshpande N, Narayan P. Establishment and characterization of an immortalized but non-transformed human prostate epithelial cell line: BPH-1. *In Vitro Cell Dev Biol Anim.* 1995; 31:14–24. [PubMed: 7535634]
23. Ustach CV, Taube ME, Hurst NJ Jr, Bhagat S, Bonfil RD, Cher ML, et al. A potential oncogenic activity of platelet-derived growth factor d in prostate cancer progression. *Cancer Res.* 2004; 64:1722–9. [PubMed: 14996732]
24. Najy AJ, Day KC, Day ML. ADAM15 supports prostate cancer metastasis by modulating tumor cell-endothelial cell interaction. *Cancer Res.* 2008; 68:1092–9. [PubMed: 18281484]
25. Bronzert DA, Pantazis P, Antoniadis HN, Kasid A, Davidson N, Dickson RB, et al. Synthesis and secretion of platelet-derived growth factor by human breast cancer cell lines. *Proc Natl Acad Sci U S A.* 1987; 84:5763–7. [PubMed: 3039506]
26. Johnsson A, Heldin CH, Wasteson A, Westermark B, Deuel TF, Huang JS, et al. The c-sis gene encodes a precursor of the B chain of platelet-derived growth factor. *EMBO J.* 1984; 3:921–8. [PubMed: 6329745]
27. Kaetzel DM Jr, Morgan D 3rd, Reid JDt, Fenstermaker RA. Site-directed mutagenesis of the N-linked glycosylation site in platelet-derived growth factor B-chain results in diminished intracellular retention. *Biochim Biophys Acta.* 1996; 1298:250–60. [PubMed: 8980650]
28. Rozengurt E, Stroobant P, Waterfield MD, Deuel TF, Keehan M. Platelet-derived growth factor elicits cyclic AMP accumulation in Swiss 3T3 cells: role of prostaglandin production. *Cell.* 1983; 34:265–72. [PubMed: 6192934]
29. Ouwens DM, de Ruyter ND, van der Zon GC, Carter AP, Schouten J, van der Burgt C, et al. Growth factors can activate ATF2 via a two-step mechanism: phosphorylation of Thr71 through

- the Ras-MEK-ERK pathway and of Thr69 through RalGDS-Src-p38. *EMBO J.* 2002; 21:3782–93. [PubMed: 12110590]
30. LaRochelle WJ, Jeffers M, McDonald WF, Chillakuru RA, Giese NA, Lokker NA, et al. PDGF-D, a new protease-activated growth factor. *Nat Cell Biol.* 2001; 3:517–21. [PubMed: 11331882]
  31. Lin CY, Tseng IC, Chou FP, Su SF, Chen YW, Johnson MD, et al. Zymogen activation, inhibition, and ectodomain shedding of matriptase. *Front Biosci.* 2008; 13:621–35. [PubMed: 17981575]
  32. Uhland K. Matriptase and its putative role in cancer. *Cell Mol Life Sci.* 2006; 63:2968–78. [PubMed: 17131055]
  33. Uutela M, Wirzenius M, Paavonen K, Rajantie I, He Y, Karpanen T, et al. PDGF-D induces macrophage recruitment, increased interstitial pressure, and blood vessel maturation during angiogenesis. *Blood.* 2004; 104:3198–204. [PubMed: 15271796]
  34. Owen KA, Qiu D, Alves J, Schumacher AM, Kilpatrick LM, Li J, et al. Pericellular activation of hepatocyte growth factor by the transmembrane serine proteases matriptase and hepsin, but not by the membrane-associated protease uPA. *Biochem J.* 2010; 426:219–28. [PubMed: 20015050]
  35. Li H, Fredriksson L, Li X, Eriksson U. PDGF-D is a potent transforming and angiogenic growth factor. *Oncogene.* 2003; 22:1501–10. [PubMed: 12629513]
  36. Huang W, Fridman Y, Bonfil RD, Ustach CV, Conley-Lacomb MK, Wiesner C, et al. A novel function for platelet-derived growth factor D: induction of osteoclastic differentiation for intraosseous tumor growth. *Oncogene.* 2011 Dec 12. Epub ahead of print.
  37. Su YC, Han J, Xu S, Cobb M, Skolnik EY. NIK is a new Ste20-related kinase that binds NCK and MEKK1 and activates the SAPK/JNK cascade via a conserved regulatory domain. *EMBO J.* 1997; 16:1279–90. [PubMed: 9135144]
  38. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell.* 2000; 100:57–70. [PubMed: 10647931]
  39. Wang Z, Kong D, Banerjee S, Li Y, Adsay NV, Abbruzzese J, et al. Down-regulation of platelet-derived growth factor-D inhibits cell growth and angiogenesis through inactivation of Notch-1 and nuclear factor-kappaB signaling. *Cancer Res.* 2007; 67:11377–85. [PubMed: 18056465]
  40. Xu L, Tong R, Cochran DM, Jain RK. Blocking platelet-derived growth factor-D/platelet-derived growth factor receptor beta signaling inhibits human renal cell carcinoma progression in an orthotopic mouse model. *Cancer Res.* 2005; 65:5711–9. [PubMed: 15994946]
  41. Zhao L, Zhang C, Liao G, Long J. RNAi-mediated inhibition of PDGF-D leads to decreased cell growth, invasion and angiogenesis in the SGC-7901 gastric cancer xenograft model. *Cancer Biol Ther.* 2010; 9:42–8. [PubMed: 20087065]
  42. Liu J, Liao S, Huang Y, Samuel R, Shi T, Naxerova K, et al. PDGF-D improves drug delivery and efficacy via vascular normalization, but promotes lymphatic metastasis by activating CXCR4 in breast cancer. *Clin Cancer Res.* 2011; 17:3638–48. [PubMed: 21459800]
  43. List K, Bugge TH, Szabo R. Matriptase: potent proteolysis on the cell surface. *Mol Med (Cambridge, Mass).* 2006; 12:1–7. [PubMed: 16838070]
  44. Bugge TH, List K, Szabo R. Matriptase-dependent cell surface proteolysis in epithelial development and pathogenesis. *Front Biosci.* 2007; 12:5060–70. [PubMed: 17569630]
  45. Wu SR, Cheng TS, Chen WC, Shyu HY, Ko CJ, Huang HP, et al. Matriptase is involved in ErbB-2-induced prostate cancer cell invasion. *Am J Pathol.* 2010; 177:3145–58. [PubMed: 20971737]
  46. Rao K, Goodin S, Levitt MJ, Dave N, Shih WJ, Lin Y, et al. A phase II trial of imatinib mesylate in patients with prostate specific antigen progression after local therapy for prostate cancer. *Prostate.* 2005; 62:115–22. [PubMed: 15389797]
  47. Najy AJ, Jung YS, Won JJ, Conley-Lacomb MK, Saliganan A, Kim CJ, et al. Cediranib inhibits both the intraosseous growth of PDGF D-Positive prostate cancer cells and the associated bone reaction. *Prostate.* 2011 Dec 27. Epub ahead of print.

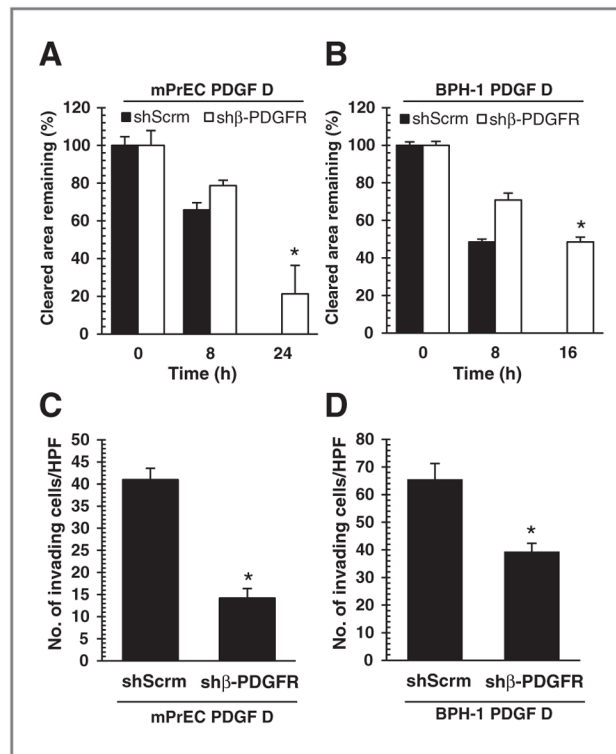
**Figure 1.**

PDGF D is a more effective inducer of prostate epithelial cell migration and invasion than PDGF B. Vector (Hygro or Neo), PDGF B or PDGF D was transfected into mPrECs; A) or human BPH-1 cells (B), overexpression was assessed via RT-PCR or immunoblotting analysis using whole-cell lysate for PDGF B or conditioned media for PDGF D. GAPDH was used as a loading control for whole-cell lysates and Ponceau S for conditioned media. mPrEC (C) or BPH-1 (D) cells were used to carry out a scratch migration assay and data plotted as the remaining cleared area. In panel C, \*,  $P < 0.05$ , comparing Hygro versus PDGF B. \*\*,  $P < 0.05$ , Neo versus PDGF D. Matrigel invasion of mPrEC (E) or BPH-1 (F) cells. Brackets above bars represent statistical significance of  $P < 0.05$ . Bars represent the mean of the experiment carried out in triplicates  $\pm$  SD.



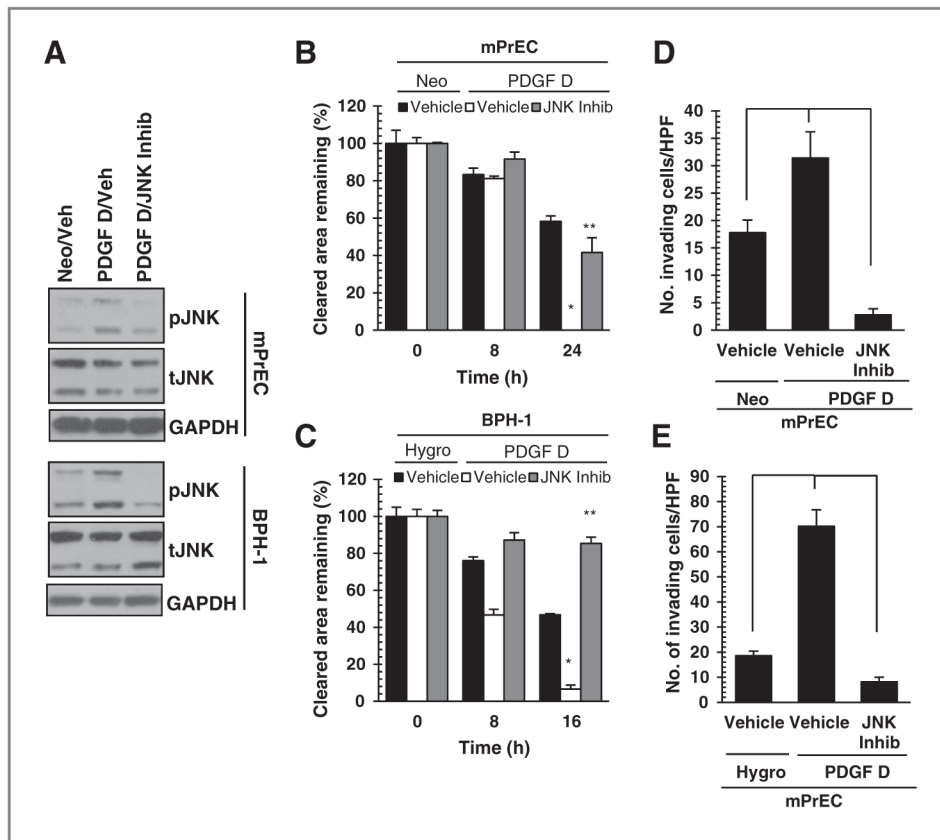


**Figure 2.** PDGF D-specific signaling activates the JNK pathway. PDGF B- and PDGF D-overexpressing mPrEC (A) and BPH-1 (B) cells were serum starved for 48 hours and whole-cell lysate harvested to assess the MAPK signaling cascade, as indicated. BPH-1 (C) and mPrE cells (D) were used to further characterize upstream and downstream components of the JNK pathway as indicated. E, PDGF D mPrEC or BPH-1 cells were transduced with scrambled or  $\beta$ -PDGFR (sh $\beta$ R) shRNA.  $\beta$ -PDGFR knockdown was assessed via RT-PCR and its effects on Erk and JNK activation.

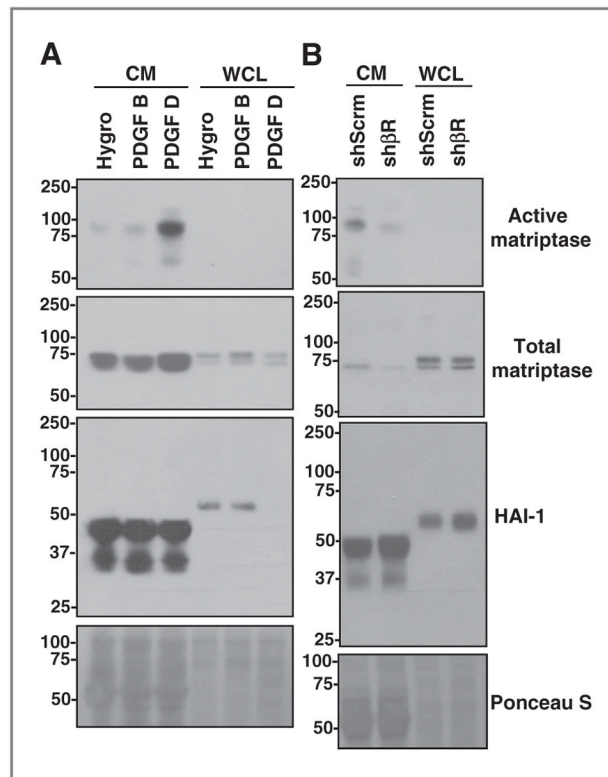


**Figure 3.**

$\beta$ -PDGFR is essential for PDGF D–mediated epithelial cell migration and invasion. PDGF D mPrEC (A) or BPH-1 (B) cells transduced with scrambled or  $\beta$ -PDGFR (sh $\beta$ R) shRNA were used for scratch migration assay. Matrigel invasion was carried out in PDGF D mPrEC (C) or BPH-1 (D) cells expressing shRNA against  $\beta$ -PDGFR. Bars represent the mean of the experiment carried out in triplicates  $\pm$  SD. \*,  $P < 0.05$ .

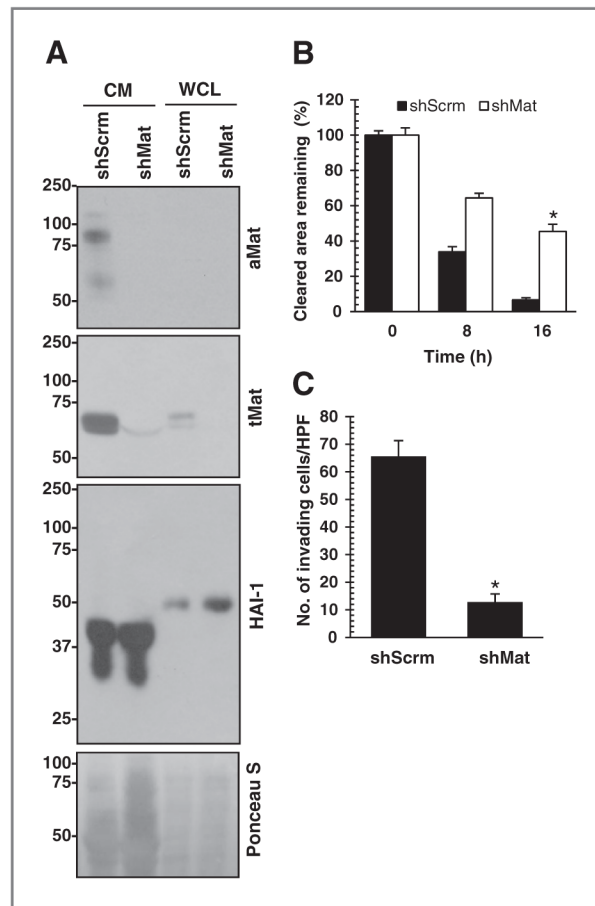
**Figure 4.**

Inhibition of JNK abrogates PDGF D-enhanced prostate epithelial cell motility and invasion. A, PDGF D mPrEC or BPH-1 cells were treated with 2.5  $\mu\text{mol/L}$  JNK inhibitor and JNK activity was monitored via immunoblotting. Scratch migration assay was assessed in response to JNK inhibition using control and PDGF D mPrEC (B) and BPH-1 (C) cells. Matrigel invasion assay of control and PDGF D expressing mPrEC (D) and BPH-1 (E) cells was carried out in response to JNK inhibition. Bars represent the mean of the experiment carried out in triplicates  $\pm$  SD. \*\*,  $P < 0.05$  comparing vehicle-treated vector control (Neo or Hygro) versus vehicle-treated PDGF D. \*\*,  $P < 0.05$  comparing PDGF D-expressing cells vehicle versus JNK inhib. Brackets above bars represent statistical significance of  $P < 0.05$ .



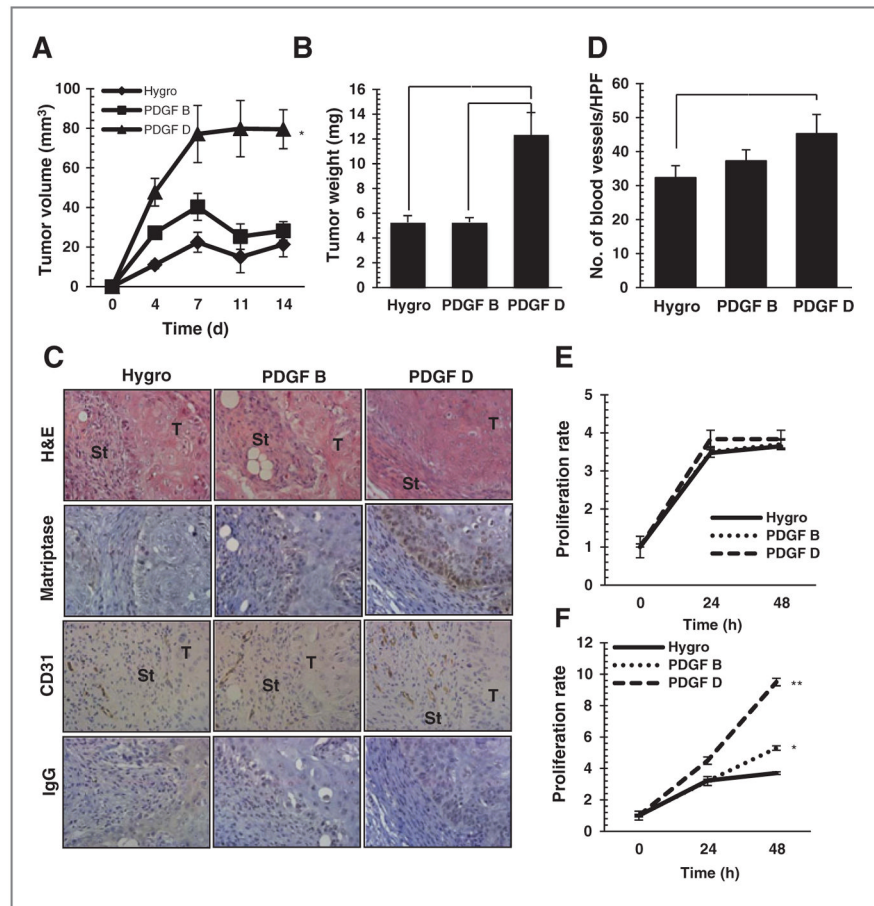
**Figure 5.**

The PDGF D-specific  $\beta$ -PDGFR signaling axis induces matriptase activation/shedding in prostate epithelial cells. A, conditioned media or WCL were collected from serum-starved vector (Hygro), PDGF B or PDGF D BPH-1 cells and analyzed for matriptase and its inhibitor, HAI-1, under nonreducing conditions. B, matriptase (active and total) and HAI-1 expression in PDGF D BPH-1 cells expressing scrambled (shScrm) or  $\beta$ -PDGFR (sh $\beta$ R) shRNA. Ponceau S was used to evaluate equal gel loading. CM, conditioned media.



**Figure 6.** Downregulation of matriptase attenuates PDGF D–mediated cell migration and invasion. A, matriptase [active (aMat) and total (tMat)] and HAI-1 expression in conditioned media or WCL in PDGF D BPH-1 cells expressing scrambled (shScrm) or matriptase (shMat) shRNA. Ponceau S was used to evaluate equal gel loading. Wound healing (scratch) cell migration (B) and Matrigel invasion (C) were assessed in PDGF D BPH-1 cells expressing scrambled or matriptase shRNA. Bars represent the mean of the experiment carried out in triplicates  $\pm$  SD. \*,  $P < 0.05$ . CM, conditioned media.





**Figure 7.** PDGF D enhances BPH-1 tumorigenesis more effectively than PDGF B. A, vector (Hygro), PDGF B, or PDGF D BPH-1 cells were injected subcutaneously and tumor volume monitored. \*,  $P < 0.05$ . B, tumor weight analysis of BPH-1 subcutaneous tumors. Bars represent the mean of the tumor weight  $\pm$  SD. Brackets above bars represent statistical significance of  $P < 0.05$ . C, hematoxylin and eosin (H&E) staining and immunohistochemical (IHC) analysis of matritase, CD31 and control IgG antibody are shown. Histologic images were obtained at 400 $\times$  magnification. T, tumor; St, stroma. D, number of tumor blood vessels was quantified using the CellSens Dimension software. Bars represent the mean value of the number of blood vessels per HPF  $\pm$  SD. Brackets above bars represent statistical significance of  $P < 0.05$ . BPH-1 Vector (Hygro), PDGF B or PDGF D BPH-1 cells were grown in serum-free media (E) or in coculture with NIH3T3 cells (F) and BPH-1 cell proliferation monitored via Trypan blue exclusion assay. \*,  $P < 0.05$  comparing vector (Hygro) versus PDGF B BPH-1. \*\*,  $P < 0.05$  comparing vector (Hygro) and PDGF B versus PDGF D BPH-1.