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PEDF and IL-6 CONTROL PROSTATE NEUROENDOCRINE DIFFERENTIATION VIA FEED-FORWARD MECHANISM

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Abstract

Pigment epithelium-derived factor (PEDF) promotes differentiation and survival of neuronal cells and expands the adult neuronal stem cell niche. In the prostate, PEDF is suppressed by androgen, with unclear physiological consequences. Ectopic and endogenous PEDF cause neuroendocrine differentiation of prostate cancer cells, manifested by neurite-like outgrowths and expression of chromogranin A. The trans-differentiated cells expressed both axonal and dendritic markers, as was ascertained by immunoblotting specific markers. Neuroendocrine cells formed multiple synaptophysin-positive protrusions resembling dendritic spines, and vesicles containing serotonin, pointing to a possibility of synapse formation. Interleukin-6 (IL-6), a known trans-differentiating agent, induced PEDF secretion. Moreover, PEDF neutralizing antibodies abolished trans-differentiation of IL-6 treated cells, suggesting an autocrine loop. Neurogenic events were independent of cyclic AMP. Instead, PEDF activated, in this order, RhoA, NFκB and Stat3. Inhibitors of Rho, NFκB or STAT pathways abolished differentiation and synapse formation. Additionally, NFκB activation caused IL-6 expression. Thus we discovered that NFκB controls formation of neuronal communications in the prostate due to PEDF and defined a feed-forward loop where NFκB induction elicits Stat3 activation and pro-differentiating IL-6 expression causing further expansion of the neuroendocrine communications. Our findings point to the role of NFκB and PEDF in coordinated prostate development.

Keywords

neuroendocrine differentiation; pigment epithelium-derived factor; NFκB; prostate cancer

INTRODUCTION

Pigment epithelium-derived factor (PEDF), a multi-functional secreted protein, controls eye development, protects the components of neural retina, and inhibits angiogenesis. PEDF is also important for differentiation, and survival in the nervous system and stimulates expansion of

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the stem cell niche in the brain^{1, 2}. In mouse prostate and in human prostate cancer (PCa) PEDF is negatively regulated by androgen³, moreover, PEDF-null mice develop epithelial hyperplasia in the prostate³. However, PEDF precise function in the prostate remains unclear. We recently showed that PEDF induces neuroendocrine differentiation of the PCa cells (NED) and identified its 44-mer N-terminal fragment responsible for this function⁴. In the normal prostate, NE cells reside among acinar and ductal secretory epithelium, they are terminally differentiated, post-mitotic, androgen-refractory, and produce serotonin and thyroid-stimulating hormone⁵. In PCa, NED is considered an adverse feature associated with increasing grade, decreased survival, and progression to androgen-independent phenotype⁶. The implications of the NED for PCa are not completely clear: while purely NE tumors are extremely aggressive, partial (focal) NED is insufficiently studied to be used as predictive marker⁷. Co-implantation of NE-10 cells from mouse NE tumors enhances the take of non-NE LNCaP⁸. However, these cells originate from TRAMP mice, where tumorigenesis is driven by Tag viral oncogene. In contrast, invasive metastatic PCa in mice caused by prostate-specific inactivation of the proper tumor suppressor, PTEN, lacks NED features⁹. IL-6, melatonin and genistein, which cause transdifferentiation, also delay PCa tumor growth in mouse models^{10–12}. In culture, NE cells secrete factors, which may induce PCa cell proliferation directly or via unlawful activation of the androgen receptor¹³. On the other hand, trans-differentiated cells can slow the growth of the epithelial PCa cells on contact and via secreted factors¹⁰. In concert, earlier data by us and others, show that transdifferentiation due to PEDF or IL-6 fails to promote tumor progression and opposes the growth of PCa xenografts^{4, 10}.

Here we report that PEDF induced NED of PCa cells accompanied by neurite outgrowth and chromogranin A (ChrA) expression. PEDF also caused PCa cells to express neuronal markers, Tau1 and MAP-2. PEDF-treated NE cells formed multiple contacts, dendritic spines and serotonin-positive vesicles suggestive of synapse formation and cell-cell communications. We found that PEDF-dependent NED required activation of RhoA, Stat3 and NFκB, which increased IL-6 mRNA. IL-6, in turn, enhanced PEDF production. Thus, we demonstrate that PEDF induces NED and define a molecular feed-forward loop that maintains and enhances this process.

MATERIALS AND METHODS

Cells and Reagents

The human PCa lines LNCaP, PC-3, DU145, and prostate epithelial strain, RWPE-1 (ATCC, Manassas, VA) were maintained in RPMI1640 (LNCaP); Ham's F12K (PC-3); Eagle's MEM/Earle's BSS (1:1), 1.5 g/L NaHCO₃, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate (DU145) and keratinocyte medium, 5 ng/ml EGF, 0.05 mg/ml bovine pituitary extract (RWPE-1). All contained 2mM L-glutamine, 10% FBS, 1% pen/strep (GIBCO, Carlsbad, CA). PC-3 expressing GFP were from Dr. B. Jimenez (University of Madrid). PC-3 expressing PEDF and the 44-mer were generated previously⁴.

IL-6 and forskolin (Fsk) were from Sigma (St Louis, MO); PEDF was from BioProducts (Middletown, MD); The following antibodies were used: Stat3 (K15), p65, and TATA binding protein (TBP) (BD Transduction Labs, Franklin Lakes, NJ); GAPDH (Chemicon, Temecula, CA); Tau-1 (Dr. L. Binder, Northwestern University), Synaptophysin (SVP-38, Sigma), Microtubule-Associated Protein 2 (MAP2, Invitrogen, Carlsbad, CA), and serotonin (LabVision, Fremont, CA). Secondary antibodies were from Santa Cruz (Santa Cruz, CA). cAMP antagonist, PKA inhibitor TTYADFIASGRTGRRNAIHD were from Santa Cruz; Stat3 inhibitor (Ac-PpYLKTK), BMS34-5541, an inhibitor of NFκB pathway, and Y-27632, Rho kinase inhibitor were from Calbiochem (San Diego, CA).

Conditioned media preparation

Sub-confluent cells were rinsed with PBS, incubated 4 hrs and re-fed with fresh serum-free media. CM were collected at 48 hrs, debris cleared, CM concentrated and dialyzed against PBS (Millipore centrifugal filters, 3 kDa, Fisher, Pittsburgh, PA).

NED Assay

Cells were seeded in complete media into 24-well plates (1.25×10^4 /well) overnight, re-fed with serum-free media containing test substances and incubated for indicated time periods. The NED was determined in triplicate samples in at least $3 \times 1 \text{ mm}^2$ areas per well: cells with outgrowth(s) $\geq 0.5 \text{ } \mu\text{m}$ scored as positive and % NED calculated.

Detection of PEDF, ChrA, IL-6 and camp

PEDF, IL-6 and ChrA were measured in CM using ELISA kits (Chemicon, Temecula, CA; DAKO, Carpinteria, CA for ChrA). cAMP was measured using EIA kit (R&D Systems, Minneapolis, MN). Absorbance was measured with the microplate reader and analyzed using WinSoft Max 2.35 software (Molecular Devices, Sunnyvale, CA).

Preparation of nuclear extracts

The cells were lysed in hypotonic buffer (50 mM NaCl, 10 mM HEPES pH 8.0, 500 mM sucrose, 1 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine, 0.2% TX-100, $200 \mu\text{l}/5 \times 10^6$ cells) with protease/phosphatase inhibitors, nuclei precipitated; washed in 50 mM NaCl, 10 mM HEPES pH 8.0, 25% glycerol, 0.1 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine, resuspended in $50 \mu\text{l}$ hypertonic buffer (350 mM NaCl, 10 mM HEPES pH8.0, 25% glycerol, 0.1 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine), incubated 30 min at 4°C , and cleared by centrifugation.

Western Blot Analysis

Cells lysates were prepared in ice-cold RIPA buffer (50mM Tris-HCl, pH 7.4, 1% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, and 1 mM NaF) and cleared (12,000g, 4°C , 10 min). Whole cell lysates or nuclear extracts ($20 \mu\text{g}/\text{lane}$) were resolved by denaturing SDS-PAGE, transferred to PVDF membranes and probed with appropriate antibodies. For neuronal markers, cells were lysed in loading buffer (0.5M Tris-HCl, glycerol, 10% SDS, bromophenol blue). Loading was assessed by immunoblot for GAPDH (total extracts) or TBP (nuclear extracts). **Rho pull-down assay** was performed using E-Z Detect Rho Activation kit (Pierce Chemicals, Rockford, IL) according to the manufacturer's instructions.

Immunostaining

PC-3 cells were grown on coverslips, fixed in 4% formaldehyde (Sigma) in PBS or ice-cold 80% methanol (Fisher), permeabilized 5–20 min with 0.5%–1% Triton X-100 in PBS, blocked in 10% FCS or horse serum and incubated with primary antibodies (4°C overnight). Synaptophysin mAb were diluted 1:200, serotonin mAb was pre-diluted. The slides were washed with PBS and incubated with FITC-conjugated anti-mouse IgG (1:200) or Fab 2 anti-goat IgG (1:300), 1 hr, room temperature. The coverslips were mounted (Fluoromount-G, Southern Biotech, Birmingham, AL) and fluorescent images taken using Nikon Diaphot-2000 microscope.

RT-PCR analysis

RNA was isolated with RNeasy kit (Quiagen, Valencia, CA), converted into cDNA (MMTV reverse transcriptase, Amersham, Piscataway, NJ) and amplified with IL-6 primers

ATGAACTCCTTCTCCACAAGC-3' and 5'-TGGACTGCAGGAACCTCCTT -3' (610-bp product) and with β -actin primers, to equalize cDNA input.

Statistical Analysis

All measurements were performed in quadruplicate, each experiment repeated at least twice. Numerical data are reported as mean $\pm$ SEM. Statistical significance was evaluated using non-paired Student's *t* test or one-way ANOVA, if multiple groups were compared. A *P* value below 0.05 was considered significant.

RESULTS

PEDF induced neurite outgrowths and ChrA secretion in PCa cells

PEDF overexpression was previously reported to cause NE morphology in PC-3 cells ⁴. In serum-deprived LNCaP cells recombinant human PEDF caused dose-, and time-dependent morphology changes consistent with NED (Fig. 1A). The effect of PEDF was comparable to that of other trans-differentiating factors, IL-6 and Forskolin (Fsk) (Fig. 1B). We also measured NED marker, ChrA in conditioned media of PEDF-treated cells. Similar to IL-6 and Fsk, PEDF caused dose-dependent increase in ChrA secretion by LNCaP cells (Fig. 1C). Interestingly, PEDF caused neurite outgrowths and increased ChrA in multiple PCa cell lines, but not in RWPE-1, a normal prostate epithelial strain (Fig. 1D, E).

IL-6 induced PEDF secretion by PCa cells

Treatment with IL-6, but not Fsk, caused LNCaP and PC-3 cells to secrete PEDF (Fig. 1F and data not shown). PEDF was at least in part responsible for the NED caused by IL-6, as PEDF antibody attenuated NE morphology (Fig. 1G) and ChrA secretion (Fig. 1H) elicited by PEDF and IL-6, but not by Fsk.

PEDF promoted neuronal features in trans-differentiated cells

To analyze NED by PEDF, and to exclude the role of androgen receptor, we chose a PC-3 clone with uniform epithelial morphology (Fig. 2A, panel a). After 3–6 days of PEDF treatment, the cells formed structures resembling axons, growth cones, and dendrites (Fig. 2A panel b and c). In addition, PEDF caused multiple cell-cell contacts resembling synapses (Fig. 2B and C). To facilitate imaging, we used PC-3 cells stably expressing GFP (Fig. 3D). Both exogenous PEDF and its neurotrophic 44-mer fragment caused neurite-like outgrowths in PC3-GFP cells (Fig. 2E, F).

PEDF and its neurotrophic 44-mer fragment induced neuronal markers and serotonin

Consistent with our previous observations ⁴, PEDF and its neurotrophic peptide, the 44-mer caused NE morphology in PC-3 cells, while anti-angiogenic 34-mer did not (Fig. 3A). Western blot analysis and immunostaining revealed increase of neuronal markers, somatodendritic microtubule-associated protein 2 (MAP2) and axonal Tau-1, in PC-3 cells treated with PEDF and 44-mer, but not the 34-mer (Fig. 3B–D). Both MAP2 and Tau-1 localized to cell projections (Fig. 3C, D). PC-3 cells treated with exogenous PEDF or with synthetic 44-mer expressed synaptophysin, a synaptic marker (Fig. 3E). Furthermore, PEDF and 44-mer increased the staining for neurotransmitter serotonin (Fig. 3F). In addition, PC-3 cells expressing tetracycline-inducible PEDF or the 44-mer, when cultured with Doxycyclin, also displayed NE morphology (Fig. 3G, H), expressed synaptophysin (Fig. 3G) and formed vesicles containing serotonin (Fig. 3H).

PEDF caused RhoA-dependent induction of dendritic spines

Exogenous PEDF caused neurite-like outgrowths in PC3-GFP cells, with multiple bulbous protrusions (Fig. 4A) resembling dendritic spines, the precursors of synaptic contacts in neuronal cells¹⁴. In agreement with our hypothesis, the morphology of cell-cell contacts was consistent with synaptic morphology: moreover, the contact areas and the protrusions stained positive for synaptophysin (Fig. 4B). The numbers and size of dendritic spines are controlled via small Rho GTPases, including RhoA, Rac and Cdc42¹⁴. Pull-down assays for Rho, Rac and Cdc42 showed increase in RhoA activity in PC-3 cells treated with PEDF (Fig. 4C and data not shown). Pre-treatment with Y-27632, an inhibitor of RhoA kinase (ROCK) downstream of Rho attenuated both spine formation (Fig. 4D) and differentiation, Fig. 4E).

PEDF controls NED via Stat3 but not cAMP

Stat3 was previously identified as a mediator of transdifferentiation by IL-6¹⁵. The blockade of IL-6 effect by PEDF antibodies suggests that PEDF and IL-6 may, at least in part, share the same signaling events. Indeed, like IL-6, PEDF enhanced Stat3 expression and phosphorylation (Fig. 5A). Importantly, Stat3 inhibitor, an acetylated phospho-peptide pp-YLTKK (2.5 μ M) reduced the NED by both PEDF and IL-6 (Fig. 5B) as well as ChrA expression (data not shown). In contrast, neither PEDF, nor IL-6 elevated cAMP levels in PCa cells (Fig. 5C). Moreover, PKA inhibitor, which blocks cAMP pathway interfered with Fsk but not with PEDF or IL-6 (Fig. 5D).

NED by PEDF involved NF κ B activation and elevated IL-6

Since PEDF neuroprotection involves NF κ B¹ we investigated its role in the NED of the PCa cells. Nuclear NF κ B (both p65 and p50) increased after 30 min treatment with PEDF or IL-6 but not Fsk (Fig. 6A). STAT3 activation occurred downstream of NF κ B, as STAT3 activation was diminished by BMS-345541, an inhibitor of NF κ B activating IKK kinases, while STAT inhibitor failed to block NF κ B (Fig. 6B, C). NF κ B induction by PEDF was necessary, but not sufficient to elicit NED: BMS-345541 abolished NE differentiation by PEDF and the 44-mer (Fig. 6D, E) and ChrA induction by PEDF (Fig. 6F); however, TNF caused NF κ B activation but failed to induce NED (not shown). Non-incidentally, IL-6, an NF κ B target, was increased after 12-hour treatment with PEDF or IL-6 but not with Fsk, as was determined by semi-quantitative RT-PCR (Fig. 6E). Moreover, PEDF treatment increased secreted IL-6 protein and this increase was attenuated by PEDF antibody, or with NF κ B inhibitor, BMS-345541 (Fig. 6F). Therefore PEDF and IL-6 are likely to induce NED via feed-forward loop (Fig. 7).

DISCUSSION

Increased NE component is linked to more aggressive, hormone-refractory PCa and poor outcomes^{6, 16, 17}. Delineating NED mechanisms may thus be key in reconstructing PCa progression, factoring in the role of microenvironment. Our study demonstrates that PEDF, a protein intimately linked to prostate development³ and neuronal differentiation¹ may also maintain NED and promote the NE communications that are thought to control prostate growth and development. We previously demonstrated that autocrine PEDF supports NED; here we show that exogenous PEDF also triggers transdifferentiation of PCa cell lines. The lack of response in the normal prostate epithelial cells may be due to their terminally differentiated state. PEDF-dependent NED was independent of the androgen receptor, since it occurred both on AR-positive and AR-negative PCa cell lines. In response to PEDF, PCa cells assumed neuron-like features including Tau-1 positive axons, MAP1-positive dendrites, and synaptophysin-positive dendritic spines, the precursors of synaptic contacts¹⁴. Moreover, PEDF increased levels of the neurotransmitter serotonin, which was localized to vesicular structures. All these changes imply that PEDF promotes communications between NE cells.

Since NED is thought to ensure coordinated prostate development⁵, defective NED may explain the aberrant prostate development in PEDF-null mice³.

While several agents are known to trigger NED of PCa cells lines *in vitro*, the underlying molecular events are poorly defined. NED can be induced by the cAMP agonists, via G-protein cascades and by IL-6 via JAK-Stat pathway preceded by G1 arrest¹³. It can be initiated by androgen withdrawal and suppressed by constitutively active androgen receptor. Significantly, androgen receptor interacts with and blocks gp130, an IL-6 receptor¹⁸, thus androgen withdrawal and IL-6 may trigger the same pathway. Finally, male-specific protocadherin activates Wnt to elicit NED¹⁹. Our study shows that in PCa cells PEDF activated, in this order, RhoA, NFκB and Stat3. All of these were critical for PEDF action since their respective inhibitors attenuated NED. On the other hand, we showed that PEDF upregulates IL-6 in PCa cells at mRNA level via NFκB-dependent mechanism. NFκB has been implicated in neuronal survival and differentiation¹, moreover, NFκB family members contribute to neuronal plasticity²⁰. PEDF also stimulates chemokine production by glia via NFκB pathway¹. Conversely, in the endothelial cells PEDF suppresses IL-6 production, suggesting that neurotrophic and anti-angiogenic PEDF functions are exerted via distinct epitopes. Surprisingly, we also found that IL-6 upregulated PEDF secretion by PCa cells: this event was critical for subsequent NED since PEDF antibodies attenuated transdifferentiation by IL-6. Thus it appears that PEDF and IL-6 form a feed-forward mechanism, which maintains steady-state NED levels. PEDF-dependent NED and some of the accompanying molecular changes were recapitulated by PEDF 44-mer frafment, but not by its 34-mer anti-angiogenic epitope, suggesting that PEDF acts on PCa cells via its “neurotrophic” receptor⁴.

In summary, we describe novel PEDF function in the prostate where its effect is similar to that in neuroblastoma and retinoblastoma cells¹. Doll and collaborators showed that PEDF controls prostate development, where its expression is negatively regulated by testosterone³. Our data indicate that high PEDF levels resulting from androgen ablation may cause expansion of the NE component and thus influence both prostate development and PCa progression. It has been postulated that normal NE cells coordinate growth and differentiation, in developing prostate and secretory function in mature gland. It is possible that transformed NE or NE stem cells form aggressive NE tumors, while focal NED has no tumorigenic effect. Further studies of NED by PEDF and other agents will shed more light on this problem and dictate the use of NED promoting agents, like PEDF, or NED inhibitors for prevention and treatment of hormone-refractory disease.

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Abbreviations

cAMP	cyclic Adenosine monphosphate
ChrA	Chromogranin A
ELISA	Enzyme-Linked Immunosobent Assay
FBS	fetal bovine serum
Fsk	Forskolin
IL-6	Interleukin-6

NE	Neuroendocrine
NED	Neuroendocrine Differentiation
NFkB	Nuclear Factor Kappa Beta
PCa	Prostate Cancer
PEDF	Pigment Epithelial-Derived Factor

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Figure 1. Trans-differentiation of the prostate epithelium by PEDF, IL-6 and Forskolin
(A) LNCaP cells were treated with increasing concentrations of PEDF for 3 and 6 days, where indicated, and percent of differentiated cells calculated and compared to untreated control.
(B) LNCaP cells were treated 6 days with fixed concentrations of PEDF, IL-6 and Fsk and compared to untreated control. Percent of cells with NE morphology is shown. **(C)** LNCaP cells were treated 4 days with PEDF (5nM), IL-6 (2nM) and Fsk (25nM), CM collected after subsequent days and ChrA measured by ELISA. The comparison is with untreated control.
(D) Benign prostate epithelial strain (RWPE-1), or indicated prostate cancer cell lines were treated 6 days with 5 nM PEDF, percent cells with NE morphology calculated and compared to untreated control. **(E)** LNCaP cells were treated as in (D), CM collected, ChrA measured and compared to untreated control. **(F)** LNCaP cells were treated 4 days with IL-6 or Fsk, CM collected as in (C), PEDF measured by ELISA and compared to untreated control. **(G, H)** LNCaP cells were treated with 5 nM PEDF, 2 nM IL-6, or 25 nM Fsk. PEDF neutralizing antibody or isotype-matched control antibodies (rabbit IgG) were added where indicated. NED

was scored as % cells with NE morphology (**G**) or ChrA secretion measured as in (C) (**H**) and PEDF and control antibody treatments compared. **, $P<0.01$; ***, $P<0.001$.

Figure 2. Characterization of PEDF-driven NED

(A) Examples of the NE morphology in PC-3 cells treated 72 hours with 5 nM PEDF. Note uniform shape of the untreated cells (a) and neurite-like processes in PEDF-treated cells (b, c). (B, C) Contacts and processes formed by PEDF-treated PC-3 cells. Note prominent, axon-like outgrowths (empty arrows) and synapse-like contacts (filled arrows (B), structures resembling growth cones (filled arrows, C) and spine-like protrusion (empty arrows, C). (D-H) NED of GFP-expressing PC-3 cells treated with 5 nM PEDF. The cells were treated 72 hours and photomicrographs taken using fluorescence microscope. Note increased number of processes (D-F) and long axon-like structures (B, C, D and F) after PEDF treatment.

Figure 3. Expression of neuronal markers by PEDF-treated PCa cells

(A) PC-3 cells were treated 48 hours with PEDF and two synthetic peptides, the anti-angiogenic 34-mer and the neurotrophic 44-mer (100 nM each). Cell morphology was observed by phase contrast microscopy (20X magnification). (B) Whole cell lysates were analyzed by Western blot for MAP2 and Tau-1. The blots were re-probed for tubulin to assess loading. Note the induction of both markers by PEDF and the 44-mer, but not the 34-mer. (C-F) PC-3 cells were treated with PEDF or synthetic 44-mer and stained for Tau-1 (C), MAP-2 (D), synaptophysin (E) or Serotonin (F). (G, H) PC-3 cells expressing tetracycline-inducible PEDF or the 44-mer were treated with Doxycycline (Dox) to induce conditional expression. The cells were stained with antibodies for Synaptophysin (G) or Serotonin (H).

Figure 4. PEDF-induced NED involved Rho-dependent formation of dendritic spines

(A) GFP-expressing PC-3 cells were treated with PEDF and observed at 60X magnification. Note the structures with the appearance of dendritic spines (inset, right) lacking in control cells (inset, left). (B) PEDF-treated PC-3 cells were stained for synaptophysin and observed at high magnification: note strong positive staining of the spine-like protrusions. (C) Pull-down assay for active RhoA. Note increase in the Rho-GTP in PEDF-treated cells compared to the vehicle-treated control (0.1% DMSO). (D) PC-3 cells were treated with 5 nM PEDF in the presence of Y-27632, an inhibitor of RhoA target ROCK (ROCK-i), or vehicle DMSO and stained for synaptophysin. Note the absence of synaptophysin-positive spines in the presence of ROCK inhibitor. (E) Y-27632 reversed NED by PEDF. Ctr, vehicle control (DMSO); ROCK-i, Y-27632.

Figure 5. PEDF-dependent NED involved STAT3 but not cAMP

(A) PC-3 cells treated for 2 hours with PEDF (5nM), IL-6 (2nM) or Fsk (25nM) were analyzed by Western blot with antibodies for total and phosphorylated Stat3 (pSTAT3). (B) The cells were pre-treated for 1 hour, where indicated, with Ac-PpYLKTK (Stat-i) and treated 72 hours with PEDF, IL-6 or Fsk (concentrations as in A). NE differentiation was scored as percent differentiated cells and Ac-PpYLKTK pre-treated cells compared with control compounds. ** - $P < 0.01$. (C) The cells were treated as in (A), and cAMP in cell lysates measured at 24 hours. *** - $P < 0.001$. (D) The cells were pre-treated with PKA inhibitor, TTYADFIASGRTGRRNAIHD (PKA-i), treated with PEDF, IL-6 or Fsk (concentrations as in (A) and NED morphology scored as in (B). ** - $P < 0.01$.

Figure 6. NFκB activation in PCa cells by PEDF

(A) PC-3 cells were treated 30 min with PEDF (5nM), IL-6 (2nM) or Fsk (25nM). The cells were harvested, fractionated and nuclear fractions analyzed by Western blot for NFκB subunits (p65/RelA and p50). The membranes were re-probed for TATA-binding protein (TBP) to assess loading and for tubulin, to assure the purity of the nuclear fractions. (B, C) PC-3 cells were treated with indicated combinations of PEDF (5nM), IL-6 (2nM) or Fsk (25nM) with TTYADFIASGRTGRRNAIHD (Stat inhibitor, Stat-i) or BMS-345541 (NFκB-i). Nuclear extracts were analyzed by Western blot for p65 and p50 (B), and whole cell lysates were probed for Stat-3 and phospho-Stat-3 (C). To assess loading of the nuclear extracts the membranes were re-probed for TBP, for the whole cell lysates we used GAPDH. The purity of the nuclear extracts was tested by re-probing membranes for tubulin. (D) PC-3 cells were subjected to the following treatments: (a) untreated control (serum-deprived); (b) 5nM PEDF; (c) 100nM 34-mer; (d) 100nM 44-mer; The same treatments were applied the the PC-3 cells pre-treated with NFκB inhibitor, BMS-345541 (NFκB-i, 5 μM, 1 hour pre-treatment). After 72 hours the cells were fixed, stained with crystal violet and photographed. (E) PC-3 cells were pre-treated 1 hr with BMS-345541, where shown, treated 72 hours with PEDF (5nM), IL-6 (2nM) or Fsk (25nM) and percent NED calculated. **, $P < 0.02$. (F) PC-3 cells were treated with PEDF (5nM), IL-6 (2nM), Fsk (25nM) or TNF-α (7ng/ml) overnight. RNA was isolated and IL-6

message assessed by semi-quantitative RT-PCR. Actin message was measured to equalize loading. Note increased IL-6 message in PEDF-treated cells and a milder increase by IL-6. **(F)** PC-3 cells were treated 72 hours with PEDF alone or in combination with PEDF neutralizing antibody or BMS-345541 (NF κ B-i). Isotype-matched mouse IgG were used as negative control. Secreted IL-6 was measured in CM by ELISA. * $-P<0.009$ compared to untreated control (IgG).

Figure 7.
Schematic representation of the PEDF signaling in the prostate cancer cells.