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Implications of pleiotrophin in human PC3 prostate cancer cell growth *in vivo*

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Pleiotrophin (PTN) is a heparin-binding growth factor with diverse functions related to tumor growth, angiogenesis, and metastasis. Pleiotrophin seems to have a significant role in prostate cancer cell growth and to mediate the stimulatory actions of other factors that affect prostate cancer cell functions. However, all studies carried out up to date are *in vitro*, using different types of human prostate cancer cell lines. The aim of the present work was to study the role of endogenous PTN in human prostate cancer growth *in vivo*. For this purpose, human prostate cancer PC3 cells were stably transfected with a plasmid vector, bearing the antisense PTN sequence, in order to inhibit PTN expression (AS-PC3). Migration, apoptosis, and adhesion on osteoblastic cells were measured *in vitro*. *In vivo*, PC3 cells were s.c. injected into male NOD/SCID mice, and tumor growth, survival rates, angiogenesis, apoptosis, and the number of metastasis were estimated. Pleiotrophin depletion resulted in a decreased migration capability of AS-PC3 cells compared with the corresponding mock-transfected or the non-transfected PC3 cells, as well as increased apoptosis and decreased adhesiveness to osteoblastic cells *in vitro*. In prostate cancer NOD/SCID mouse xenografts, PTN depletion significantly suppressed tumor growth and angiogenesis and induced apoptosis of cancer cells. In addition, PTN depletion decreased the number of metastases, providing a survival benefit for the animals bearing AS-PC3 xenografts. Our data suggest that PTN is implicated in human prostate cancer growth *in vivo* and could be considered a potential target for the development of new therapeutic approaches for prostate cancer. (*Cancer Sci* 2012; 103: 1826–1832)

Imbalance of the relative expression or/and relative activities of the factors that regulate functions of the human prostate gland leads to pathologies, such as prostate cancer. Several factors play significant role(s) in prostate cancer development, such as androgens,[1] oxidative stress,[2] and tyrosine kinases.[3–6] It has been previously shown that many of these factors regulate the expression of the growth factor pleiotrophin (PTN) by prostate cells. For example, androgens regulate PTN levels in the prostate,[7] whereas hydrogen peroxide[8] and fibroblast growth factor 2 (FGF2)[9] upregulate PTN transcription and secretion in human prostate cancer LNCaP cells through activator protein 1 activation. Interestingly, PTN mediates at least some of the actions of both hydrogen peroxide and FGF2 on prostate cancer cells, such as induction of cell proliferation and migration.[8,9]

Pleiotrophin is a heparin-binding growth factor that, together with midkine, forms a distinct family of growth factors. A role for PTN in human cancers was first suggested after the detection of PTN mRNA or/and protein in various human cancer cell lines and tumor specimens of diverse origin. Its role has since been investigated by regulating PTN expression in several different types of tumor cells and studying their growth *in vitro* and *in vivo*. In most of the studies, overexpression of PTN leads to a more malignant phenotype, whereas downregulation of PTN expression leads to decreased growth, migration, and invasion of the tumor cells *in vitro* and *in vivo* (revised in Ref. 10). Pleiotrophin seems to be involved in the growth and morphogenesis of the prostate,[7] and might have a role in prostate cancer, based on studies showing that its expression was increased in prostate cancer compared with the physiological gland or benign prostatic hyperplasia. Moreover, PTN overexpression in normal prostate epithelial PNT-1A cells induced both anchorage-dependent and -independent growth.[11] By downregulating PTN expression in the human prostate cancer cell line LNCaP, it was found that PTN was essential for LNCaP cell migration, anchorage-dependent and –independent growth, as well as their potential to activate endothelial cells and induce angiogenesis.[12] However, all these studies are *in vitro* and, to date, there is no direct evidence concerning the role of PTN in prostate cancer growth *in vivo*.

The aim of the present study was to investigate the role of endogenous PTN in human prostate cancer growth and metastasis *in vivo*. For this purpose, we chose to use androgen-independent PC3 cells, which are highly metastatic and known to grow well in immunocompromised mice *in vivo*, without the need for supporting matrices.[6,13] We found that downregulation of PTN expression in human prostate cancer PC3 cells resulted in decreased migration, increased apoptosis, and decreased adherence on osteoblastic cells *in vitro*, as well as increased apoptosis, decreased tumor growth and angiogenesis, inhibition of tumor metastasis, and increased animal survival *in vivo*.

Materials and Methods

Cell culture. The human prostate cancer epithelial cell line PC3 and the osteoblast-like cell line MG-63 (ATCC, Manassas, VA, USA) were routinely grown in RPMI-1640 or DMEM medium, respectively, supplemented with 10% FBS, 100 U/mL penicillin/100 μg/mL streptomycin, 50 μg/mL gentamycin, and 2.5 μg/mL amphotericin B. Cultures were maintained at 37°C, 5% CO_2, and 100% humidity. Cell culture reagents were from Biochrom AG (Berlin, Germany).

Stable transfection of PC3 cells. PC3 cells were seeded at 5×10^4 cells/well in 24-well plates in RPMI-1640 containing 10% FBS and antibiotics. Twenty-four hours later, transfection was carried out using pCDNA3.1 alone or pCDNA3.1 carrying

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full-length cDNA for PTN in antisense orientation[12] and Transfast Reagent (Promega, Madison, WI, USA), according to the manufacturer's instructions. Isolation of stable transfectants was carried out by selection in 600 μg/mL neomycin G418 for 30 days. Each G418-resistant clonal population was trypsinized, resuspended, and cultured in the selective medium. The AS-PTN clones secreting the lowest amount of PTN were selected (AS1-PTN and AS2-PTN). Several clones transfected with pCDNA3 alone were selected and pooled (PC-PC3). In all cases, the presence of PTN secreted in the culture medium was investigated by Western blot analysis.

Western blot analysis for PTN. The conditioned medium of equal numbers of cells was incubated overnight with 100 μL heparin–sepharose (Amersham Pharmacia Biotech, Piscataway, NJ, USA) at 4°C with continuous agitation.[12] Heparin–sepharose was washed three times with 10 mL of 20 mM HEPES pH 7.4 and 0.5 M NaCl, and twice with 10 mL of 20 mM HEPES, pH 7.4. Bound proteins were eluted with 50 μL Laemmli buffer under reducing conditions, fractionated on 17.5% SDS-PAGE and transferred to Immobilon P membranes. Blocking was carried out by incubating the PVDF membranes with 3% BSA in TBS containing 0.05% Tween 20 (TBS-T). The membranes were then incubated with anti-PTN mAb (Abnova, Taipei, Taiwan) at a dilution of 1:1000 in TBS-T for 18 h at 4°C under continuous agitation, then with HRP-conjugated rabbit anti-mouse IgG (Sigma, St. Louis, MO, USA) at a dilution of 1:5000 in TBS-T, for 1 h at room temperature under continuous agitation. Detection of immunoreactive bands was carried out using the ECL detection kit (Pierce Biotechnology Inc., Rockford, IL, USA).

Migration assay. Migration assays were carried out in 24-well microchemotaxis chambers (Corning Incorporated, Corning, NY, USA), using uncoated polycarbonate membranes with 8-μm pores.[12] Serum-starved cells were harvested and resuspended at a concentration of 10^5 cells/0.1 mL in serum-free medium containing 0.25% BSA. The bottom chamber was filled with 0.6 mL of the same medium containing the tested substances. The upper chamber was loaded with 0.1 mL medium containing cells and incubated for 4 h at 37°C. The filters were fixed and stained with 0.33% toluidine blue solution and the cells that migrated through the filter were quantified using a grid and an Optech microscope (Optech Microscope Services Ltd, Thame, UK).

Apoptosis assay. Cells were seeded at 1×10^5 cells/well in 6-well plates. Twenty-four hours later, they were starved for 16 h, resuspended in 100 μL binding buffer and incubated with 2.5 μL Annexin V–FITC at 25°C, for 15 min. Propidium iodide stock solution (5 μL) was added followed by 200 μL binding buffer and the cells were analyzed by flow cytometry (EPICS-XL; Beckman Coulter, Nyon, Switzerland), according to the kit manufacturer's instructions (rh Annexin V/FITC kit; Bender MedSystems, Vienna, Austria).

Adhesion assay. Twenty thousand MG-63 cells were plated in each well of a 96-well plate. After 48 h, when the cells had formed a monolayer, the medium was aspirated and 30 000 PC-PC3 or AS1-PC3 cells were added to each well.[14] The cells were incubated for 2 h at 37°C, then the non-adherent cells were carefully removed and crystal violet staining was carried out as previously described.[15] The optical absorbance was measured at 630 nm.

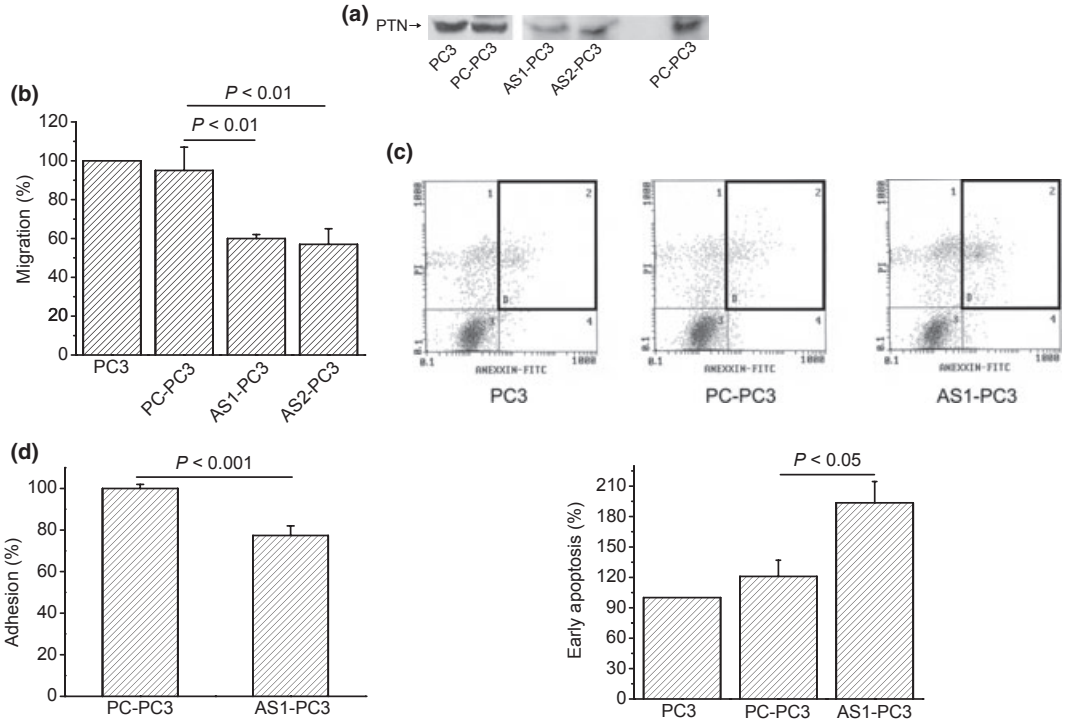

Fig. 1. (a) Secretion of pleiotrophin (PTN) by PC3 prostate cancer cells. Western blot analysis of culture media from the same number of non-transfected and stably transfected PC3 cells. (b) Migration was measured using microchemotaxis chambers. Results are expressed as the mean ± SEM percentage number of migrating cells compared with the non-transfected PC3 cells (default = 100) from four independent experiments. Migrating cells were quantified at the entire filter. (c) Representative pictures of FACS analysis of cells stained with annexin V and propidium iodide. Early apoptotic cells are those present in the D2 region. Results are expressed as the mean ± SEM percentage number of early apoptotic cells compared with the non-transfected PC3 cells (default = 100) from six independent experiments. (d) Adhesion of PC3 cells to an MG-63 cell lawn *in vitro*. Results are expressed as the mean ± SEM percentage number of adhered AS1-PC3 cells compared with PC-PC3 cells (default = 100) from three independent experiments, each carried out in duplicate.

Table 1. Percent take rate of tumors that have developed compared with the number of injected sites at various time intervals after inoculation of PC-PC3 and AS1-PC3 or AS2-PC3 cells in NOD/SCID mice

	Take rate (%)			
DPI	11	23	28	35
PC-PC3	90.0	100.0	100.0	100.0
AS1-PC3	11.1	33.3	77.8	88.9
AS2-PC3	11.1	38.9	66.7	89.0

The PC-PC3 group consisted of 10 mice; both AS-PC3 groups consisted of nine mice. Each mouse was inoculated bilaterally with cells at both rear axillas (two injections per mouse). DPI, days post inoculation of cells into mice.

Xenograft development. Male NOD/SCID (NOD.CB17 Prkdcscid) mice (Charles River Laboratories International Inc., Wilmington, MA, USA), 6–8 weeks old, were used for this study. Animals were injected s.c. with PC-PC3 or two clones of AS-PC3 cells (AS1-PC3 and AS2-PC3) at the two rear axillas (1×10^6 cells/100 μL sterile PBS/injection). Tumors were measured twice per week using a digital caliper. The formula $(a \times b^2)/2$, where a = length and b = width, was used for the calculation of tumor size. Survival, animal weight, and other changes were also recorded. After the animals were killed, autopsies were carried out and metastases were observed, counted, and photographed. Tumors (primary and metastatic) were consequently removed and embedded in paraffin for further analysis. All experiments were carried out in the animal facility of the Biomedical Research Foundation of the Academy of Athens (Code Numbers EL 25 BIO 001, EL 25 BIO 002, EL 25 BIO 003). All animals were housed and handled in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS no 123/Council Directive 86/609/EEC), as well as the current Guidelines of International Organizations (i.e. Association for the Assessment and Accreditation of Laboratory Animal Care International, and the Federation of European Laboratory Animal Science Associations) and the corresponding Greek law (Presidential Decree 160/91 in harmonization to the Council Directive 86/609/EEC).

Histochemical staining. Paraffin tissue sections (5-μm thick, placed on positively charged glass slides) from different primary tumors or metastases were stained with standard H&E, viewed in a Zeiss microscope (Carl Zeiss Microscopy GmbH, Jena, Germany), and photographed using a digital camera.

TUNEL assay. Apoptosis was determined on paraffin tissue sections using the DeadEnd Fluorometric TUNEL System (Promega), according to the manufacturer. Images were captured with a Nikon 80i Upright Microscope (Nikon Corporation, Tokyo, Japan) equipped with a Nikon Digital Sight DS-Fi1 color camera, using the MetaMorph microscopy automation and image analysis software.

Immunofluorescence. After deparaffinization of tissue sections, antigen unmasking was achieved by boiling the slides for 10 min in 1 mM EDTA pH 8.0. Sections were rinsed three times with ddH2O, washed twice with ddH2O, and once with TBS-T and blocked for 30 min with Image-iT FX signal enhancer (Invitrogen, Grand Island, NY, USA). *Griffonia simplicifolia* lectin I conjugated to rhodamine (Vector Laboratories Inc., Burlingame, CA, USA) was diluted 1:200 in TBS-T and incubated with the sections overnight at 4°C. Following incubation, sections were washed three times, 5 min each, with TBS-T and mounted in Vectashield mounting medium with Dapi (Vector Laboratories). Images were captured with a Nikon 80i Upright Microscope as described above.

Statistical analysis. The significance of variability between the results of each group and its corresponding control was determined by unpaired *t*-test.

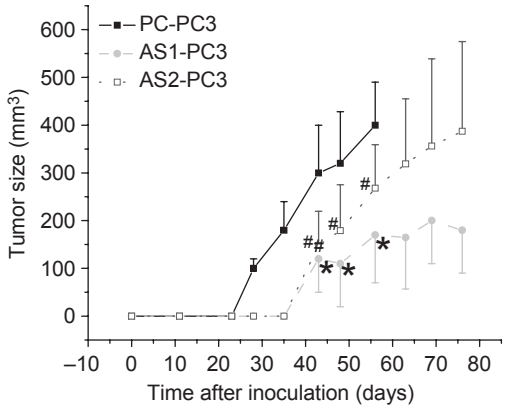

Fig. 2. Xenografts of AS1-PC3 and AS2-PC3 prostate cancer cells grew significantly slower and the size of the tumors was smaller compared with PC-PC3 xenografts at the same time point. Results are expressed as the mean ± SD of the size of the tumor. The PC-PC3 group consisted of 10 mice, while both AS-PC3 groups consisted of nine mice. Each mouse was inoculated bilaterally with cells at both rear axillas (two injections per mouse). *$P < 0.05$, AS1-PC3 *versus* PC-PC3 xenografts; #$P < 0.05$, AS2-PC3 *versus* PC-PC3 xenografts.

Table 2. Time in days that was needed for PC-PC3 and AS1-PC3 tumors to reach the size of 100 mm^3 and 200 mm^3 in NOD/SCID mice after inoculation with prostate cancer cells

	Tumor size (mm^3)		dt (100–200)
	100	200	
PC-PC3	28.0	36.0	8.0
AS1-PC3	41.1	66.5	25.4

Doubling time [dt(100–200)] is the median time interval in days required for an increase in tumor size from 100 to 200 mm^3. The PC-PC3 group consisted of 10 mice and the AS1-PC3 group of nine mice. Each mouse was inoculated bilaterally with cells at both rear axillas (two injections per mouse). Differences between the AS1-PC3 and the PC-PC3 xenografts are statistically significant ($P < 0.01$).

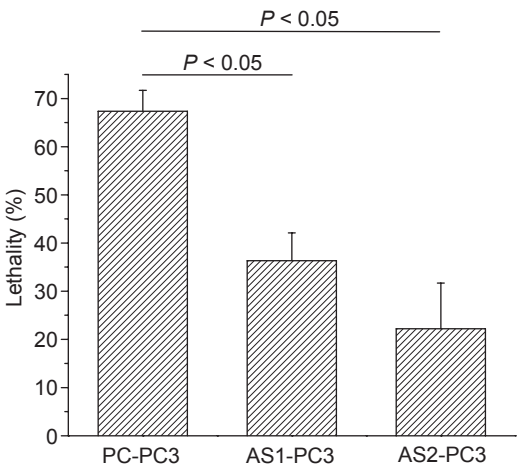

Fig. 3. Survival of NOD/SCID mice inoculated with PC3 prostate cancer cells was significantly higher in AS-PC3 compared with the PC-PC3 xenografts. Results are expressed as the mean ± SEM of the percentage of dead compared with the total number of animals from three independent experiments. Each group consisted of 5–10 mice (two bilateral tumors/mouse).

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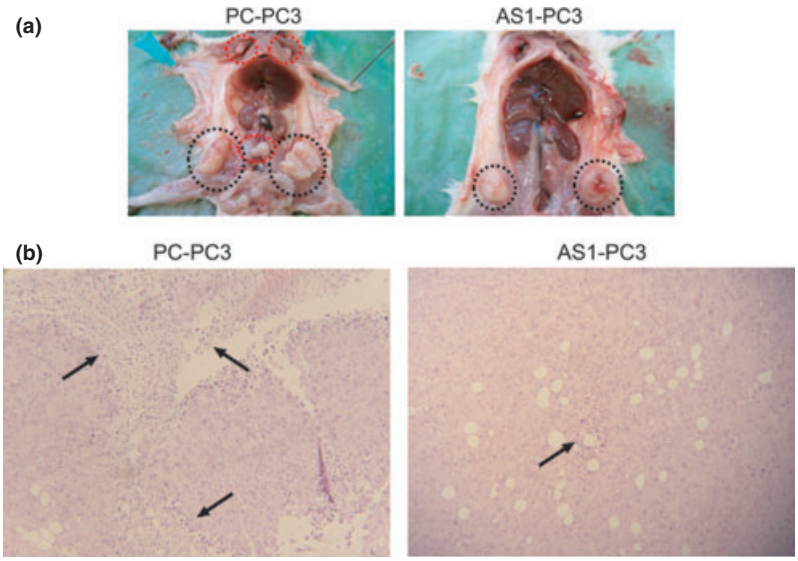

Fig. 4. (a) Representative photographs showing primary tumors at the primary sites of cancer cell inoculations (rear axillas, black circles) or metastases (front axillas and lower part of the spine behind the guts, black arrows) in NOD/SCID mice inoculated with PC3 prostate cancer cells. (b) Representative micrographs of H&E staining of paraffin sections from metastases at the lower part of the spine, showing that metastases from PC-PC3 tumors have extended necrotic areas (black arrows) compared with those from AS1-PC3 tumors. Magnification, ×10.

Results

Inhibition of PTN expression inhibits human prostate cancer cell migration and adhesion and increases apoptosis *in vitro*. Equal amounts of cell culture medium corresponding to equal numbers of cells were analyzed by Western blot analysis for PTN. As shown in Figure 1(a), AS1-PC3 and AS2-PC3 cells secreted significantly reduced amounts of PTN in their culture medium, whereas control PC-PC3 cells secreted amounts comparable with the non-transfected PC3 cells. These data indicate the efficacy of antisense PTN inhibition. PC-PC3 cell migration was comparable to that of non-transfected PC3 cells, whereas downregulation of PTN expression resulted in a significantly decreased AS-PC3 cell migration (Fig. 1b). Apoptosis was significantly increased in AS1-PC3 compared with PC-PC3 cells (Fig. 1c). Finally, AS1-PC3 cells showed significantly decreased adherence on the osteoblastic (MG-63) cell lawn compared with PC-PC3 cells (Fig. 1d).

Pleiotrophin regulates prostate cancer growth *in vivo*. Three to 4 weeks after the inoculation of PC3 cells, all mice inoculated with PC-PC3 cells developed tumors. At the same time, only one-third of the mice inoculated with either AS1-PC3 or AS2-PC3 cells developed tumors (Table 1). Further to that, 6–8 weeks after inoculation of the cells, mice inoculated with AS1-PC3 and AS2-PC3 cells developed significantly smaller tumors compared with mice inoculated with PC-PC3 cells (Fig. 2). Tumor growth in mice inoculated with both AS1-PC3 and AS2-PC3 cells showed a significant delay until week 5 post tumor injection, and even at later time points, AS1-PC3 tumors (but not AS2-PC3, data not shown) were growing at a much slower rate than PC-PC3 tumors, also evidenced by the different doubling times shown in Table 2.

Table 3. Number of metastases developed in PC-PC3 (n = 3) and AS1-PC3 (n = 4) mice inoculated with prostate cancer cells

	PC-PC3				AS1-PC3				
	1	2	3	%MS	1	2	3	4	%MS
PR	2	2	2	100	2	2	2	2	100
LS	3	1	2	100	0	0	1	1	25
FA	1	2	1	66.7	0	0	1	0	12.5

Autopsies were carried out and metastases were observed, counted, and photographed prior to removing and embedding them in paraffin for further histological analysis. Numbers in bold correspond to animals used for autopsies. The animals examined had developed tumors at both rear axillas, which were the sites of tumor cell injection and, thus, primary sites (PR) of tumor development. FA, metastases at front axillas; LS, metastases at the lower part of the spine behind the guts; %MS, percent rate of metastatic sites, calculated assuming that each primary site may give rise to one LS and one FA metastasis.

Pleiotrophin inhibition prolongs survival in prostate cancer cell mouse xenografts. Sixty days after inoculation of PC-PC3 cells into the mice, more than half of the animals died and the experiment ended. At the same time interval, only one animal inoculated with AS1-PC3 and one with AS2-PC3 cells died, whereas, 76 days post inoculation, more than half of the animals inoculated with AS1-PC3 and AS2-PC3 cells were still alive (Fig. 3), with no obvious behavioral, dietary, or weight changes observed in the living animals. These data suggest that mice inoculated with both AS1-PC3 and AS2-PC3 cells had increased survival rates compared with mice inoculated with PC-PC3 cells.

Pleiotrophin regulates prostate cancer cell metastatic potential *in vivo*. At the end of the experiment (60 days post inoculation for PC-PC3 and 76 days for AS-PC3), autopsies were carried out on the animals. The autopsies revealed that, apart from the tumors at the primary sites, tumors were also detectable at distant sites (Fig. 4a). All PC-PC3 inoculated mice developed multiple distant tumors, whereas in AS-PC3 inoculated animals, the incidence of distant tumors was quite low (Table 3; Fig. 4a). PC-PC3 metastases had extended necrotic areas, which might indicate a more aggressive cancer, whereas AS-PC3 metastases had significantly smaller necrotic areas (Fig. 4b).

Histological evaluation of PC-PC3 and AS-PC3 primary tumors. Both PC-PC3 and AS-PC3 primary tumors were malignant, with a lot of atypical, pleiomorphous, eosinophilic cells with large nuclei (Fig. 5a). Mitotic index was not significantly different between PC-PC3 and AS-PC3 tumors (Fig. S1). However, AS-PC3 primary tumors contained a significantly increased number of apoptotic cells (Fig. 5b) and a reduced number of blood vessels (Fig. 5c), compared with the PC-PC3 primary tumors.

Discussion

In the present work we showed that downregulation of endogenous PTN by stable antisense PTN expression in human prostate PC3 cancer cells resulted in inhibition of cell migration

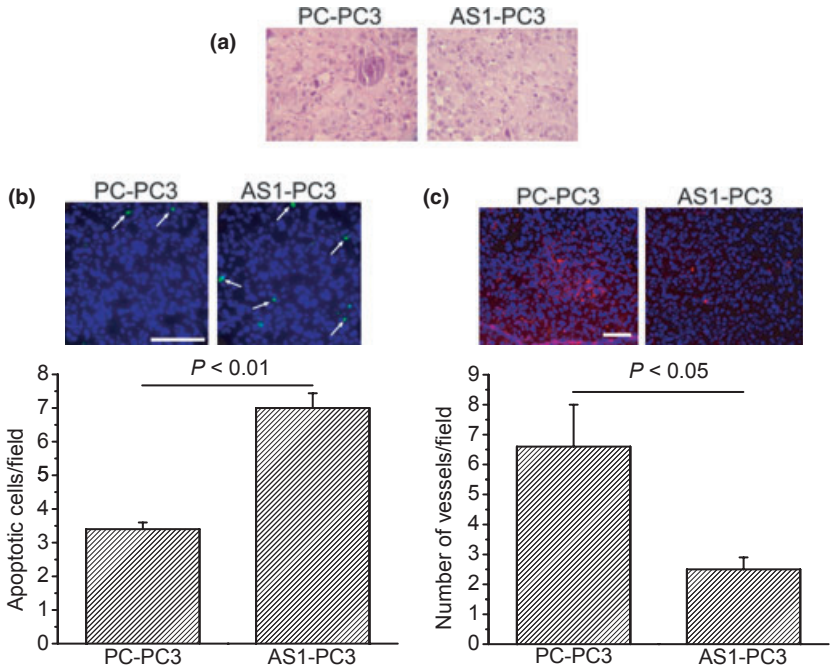

Fig. 5. (a) Haematoxylin–eosin staining of paraffin sections of primary tumors in NOD/SCID mice inoculated with PC3 prostate cancer cells. Both PC-PC3 and AS1-PC3 primary tumors were malignant with a lot of atypical, pleiomorphous, eosinophilic cells with large nuclei. Magnification, ×40. (b) Apoptosis was determined by TUNEL assay in paraffin sections from PC-PC3 and AS1-PC3 primary tumors. Tissue sections were examined by fluorescence microscopy and apoptotic cells were counted after microscope slide scanning. Upper panel, representative pictures. Apoptotic cells (green) are shown by white arrows. Scale bar = 100 μm. Lower panel, data are expressed as the number of apoptotic cells per field ± SEM. (c) Upper panel, representative pictures of immunofluorescence staining of PC-PC3- and AS1-PC3-derived primary tumors with rhodamine-labeled *Griffonia simplicifolia* lectin I (red) for blood vessels. Scale bar = 100 μm. Lower panel, data are expressed as the number of blood vessels per field ± SEM. The number of apoptotic cells and blood vessels were counted in 10 sections from three different mice (25 fields/section). Nuclei stained blue with DAPI. All images were captured and processed using identical settings.

and adhesion *in vitro*, increased apoptosis, and suppression of tumor growth, angiogenesis, and metastasis *in vivo*. These data extend previous *in vitro* studies that support a potential role of PTN in prostate cancer cell functions[11,12] to an effect of PTN in prostate cancer growth *in vivo*. To the best of our knowledge, this is the first *in vivo* study directly implicating PTN in prostate cancer growth, with the limitation that only one type of prostate cancer cell line was used. Based on the present and previous studies, it seems that PTN is a growth factor that may be significant for both hormone-dependent and -independent prostate cancer. PC3 cells are androgen-independent and highly metastatic; previous studies have been carried out in both androgen-dependent and independent prostate cancer cells.[11,12] The same notion is also supported by studies showing that expression of PTN in prostate cells is regulated by androgens,[7,11] signaling concentrations of hydrogen peroxide,[8] nitric oxide,[16] and FGF2.[9] Androgens play a significant role in hormone-dependent prostate adenocarcinoma,[1] whereas FGF2,[4] nitric oxide,[17] and other growth factors that signal through hydrogen peroxide,[18,19] also play significant role(s) in hormone-independent types of prostate cancer, which are more metastatic and difficult to treat.[20] More recently, it has been suggested that PC3 cells develop tumors that are distinct from prostate adenocarcinoma and similar to small-cell neuroendocrine carcinoma, a less frequent type of prostate cancer.[21] Pleiotrophin may be thus considered as a potential therapeutic target for this type of prostate cancer as well.

The mitotic index, as estimated by Ki67, was not found to be significantly different between PC-PC3 and AS-PC3 tumors *in vivo*, in agreement with the lack of effect of PTN in PC3 cancer cell proliferation *in vitro* (Fig. S2). However, *in vitro* cancer cell migration and *in vivo* growth and metastasis of tumors were significantly decreased in AS-PC3 cells, suggesting that the effect of PTN downregulation in prostate cancer cell migration may be more significant for the observed *in vivo* effects. Pleiotrophin-stimulated cell migration requires the presence of $\alpha_v\beta_3$,[22] which is implicated in prostate cancer cell metastasis to bone.[23,24] This supports a possible role for PTN in prostate cancer cell bone metastasis, which comes in line with our *in vitro* observation of decreased prostate cancer cell adhesion on osteoblastic cells upon PTN depletion. Besides a direct interaction with osteoblasts, it has been recently shown that human prostate cancer cells directly compete with hematopoietic stem cells for occupancy of the mouse hematopoietic stem cell niche, suggesting that the endosteal hematopoietic stem cell niche plays a central role in bone metastasis.[25] Pleiotrophin regulates hematopoietic stem cell expansion and regeneration,[26] which also depend on hematopoietic stem cell niches,[27,28] further supporting the notion that PTN might also play a role in prostate cancer bone metastasis by targeting the hematopoietic stem cell niches.

RPTPβ/ζ seems to be significant for prostate cancer cells' migration[16] and proliferation[29] *in vitro*. RPTPβ/ζ binds the intact molecule of PTN, but not PTN that lacks its carboxy-terminal domain, and is responsible for the effect of PTN in cancer cell migration.[30] Overexpression of PTN that lacks its carboxy-terminal domain acts as a dominant negative form of PTN and inhibits prostate cancer cell growth *in vitro*.[31] Similarly, a peptide that corresponds to the carboxy-terminal domain of PTN and binds to RPTPβ/ζ, inhibits prostate cancer cell response to PTN *in vitro*[29] and PC3 tumor growth *in vivo*,[32] although in the latter case it has not been shown whether its effect was solely due to endogenous PTN inhibition through RPTPβ/ζ and not interference with other implicated pathways. We have previously shown that a similar peptide inhibits the stimulatory effect of PTN on endothelial cell migration through inhibition of PTN interaction with $\alpha_v\beta_3$ integrin.[33] Integrins, and especially $\alpha_v\beta_3$, play a role in prostate cancer cell metastasis to bone.[23] It is tempting, therefore, to speculate that PTN might promote prostate cancer growth and metastasis though interaction with both RPTPβ/ζ and $\alpha_v\beta_3$, similar to its effect on cell migration *in vitro*.[22]

Angiogenesis is a hallmark of cancer and seems to play a crucial role in prostate cancer progression and metastasis.[34] Pleiotrophin secreted from human prostate LNCaP cells *in vitro* induces endothelial cell migration and tube formation on Matrigel *in vitro*, as well as angiogenesis *in vivo*[12]. The present study extends these observations, showing that PC3 prostate cancer cell-derived PTN is required for tumor-induced angiogenesis *in vivo*, supporting its significance for prostate cancer progression, metastasis, or/and prognosis.

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Interestingly, AS-PC3 cells *in vitro* and AS-PC3 tumors present with increased apoptosis compared with PC-PC3 cells and tumors, respectively, suggesting that PTN may have an antiapoptotic role. This is in line with data showing that PTN prevents apoptosis of SW-13 epithelial cells,[35] spermatocytes,[36] and human embryonic stem cells,[37] and inhibits transforming growth factor beta 1-induced apoptosis in hepatoma cell lines.[38] However, PTN potentiates cardiomyocyte apoptosis.[39] Although the reason for this discrepancy is unknown, it could be explained by the different receptors, for example, $\alpha_v\beta_3$ or RPTPβ/ζ, expressed by different types of cells, as has been described for the effect of PTN on cell migration.[22,30]

In conclusion, our data show that PTN is a rate-limiting factor in the chain of events that leads to growth, angiogenesis, and metastatic spread of prostate cancer *in vivo*, further supporting its value as a potential therapeutic target for prostate cancer.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Mitotic index of human prostate PC-PC3 and AS-PC3 xenografts.

Fig. S2. Effect of pleiotrophin (PTN) on human prostate cancer PC3 cell proliferation *in vitro*.

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