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Bone-metastatic potential of human prostate cancer cells correlates with Akt/PKB activation by α platelet-derived growth factor receptor

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Prostate adenocarcinoma metastasizes to the skeleton more frequently than any other organ. An underlying cause of this phenomenon may be the ability of bone-produced factors to specifically select disseminated prostate cancer cells that are susceptible to their trophic effects. Platelet-derived growth factor (PDGF), a potent mitogen for both normal and tumor cells, is produced in several tissues including bone, where it is synthesized by both osteoblasts and osteoclasts. Here, we show that PDGF causes a significantly stronger activation of the Akt/PKB survival pathway in bone-metastatic prostate cancer cells compared to nonmetastatic cells. Normal prostate epithelial cells and DU-145 prostate cells, originally derived from a brain metastasis, are not responsive to PDGF. In contrast, epidermal growth factor stimulates Akt to the same extent in all prostate cells tested. This difference in PDGF responsiveness depends on the higher expression of α-PDGFR in bone-metastatic compared to nonmetastatic prostate cells and the lack of α-PDGFR expression in normal and metastatic prostate cells derived from tissues other than bone. Thus, α-PDGFR expression might identify prostate cancer cells with the highest propensity to metastasize to the skeleton.
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Disseminated cancer cells may initially require local nutrients and growth factors to survive in the foreign environment of distant tissues before developing into secondary tumors and it is likely that stromal factors contribute to the tissue tropism shown by several types of tumors. Patients with advanced prostate cancer (PCa) develop metastasis in approximately 90% of cases. It is therefore possible that the bone marrow – which is the way of access to the bone for migrating cancer cells – represents a milieu particularly favorable for the growth and survival of PCa cells (Fidler, 2003).

PDGF is synthesized by both osteoblasts and osteoclasts and is integral to the regulation of the perpetual bone remodeling process (Yang *et al*., 2000; Kubota *et al*., 2002). Skeletal turnover is based on bone resorption and deposition, two processes that occur continuously during lifetime and are crucially regulated by PDGF produced within the bone matrix and its effect on osteoblast activity (Mundy, 2000). As PDGF is a potent mitogen for both normal and tumor cells (Heldin and Westermark, 1999), its availability might participate in establishing a congenial microenvironment for disseminated prostate cancer cells and the cellular susceptibility to its action could provide them with a selective survival advantage. Interestingly, the α-isoform of the PDGF receptor (α-PDGFR) is expressed by prostate cancer cells both in the primitive tumor and skeletal metastases (Fudge *et al*., 1994; Chott *et al*., 1999). However, to date there is no evidence for a role of PDGF in the activation of downstream signaling pathways that could sustain the survival of PCa cells within the bone marrow microenvironment and confer a selective advantage to PDGF-responsive cells. The aims of this study were to test whether PDGF activates survival signaling pathways in human PCa cells and ascertain if cells with different bone-metastatic potential display a significant variability in PDGF responsiveness.

We employed two sublines derived from the widely used PC3 prostate cancer cell line. Both sublines are tumorigenic when injected subcutaneously in SCID mice. However, PC3-N cells are unable to migrate through a Matrigel-coated membrane *in vitro* as well as induce metastases in SCID mice, whereas PC3-ML cells are highly invasive *in vitro* and induce skeletal metastases in more than 80% of cases (Wang and Stearns, 1991). The lack of metastatic potential of PC3-N cells seems unrelated to their ability to reach the bone marrow, because immediately after their injection in SCID mice these cells located to the skeleton as effectively as the highly metastatic PC3-ML cells (Wang and Stearns, 1991). In addition, both PC3-N and PC3-ML cells express equivalent levels of CX3CR1, a chemokine receptor that we have shown mediates their *in vitro* adhesion to human bone marrow endothelial cells and migration towards osteoblasts (Shulby *et al*.,

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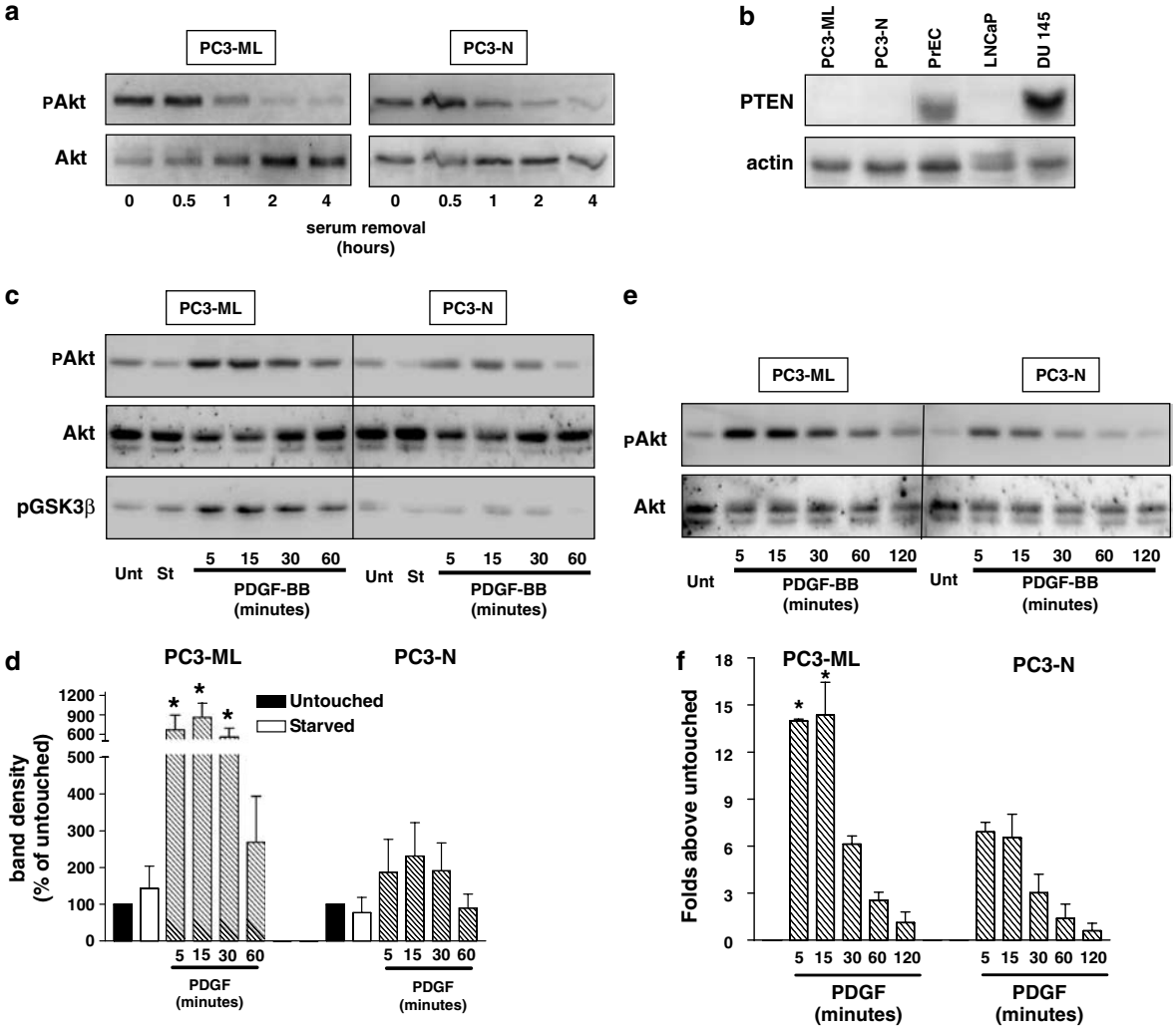


Figure 1 Invasive, bone-metastatic PC3-ML and noninvasive, nonmetastatic 3X N.I. (PC3-N) cells were isolated from the parental PC3 cell line as described previously (Wang and Stearns, 1991). (**a**) When cells were deprived of serum, levels of Akt phosphorylation slowly but progressively decreased as determined by Western blotting using an antibody directed against phosphorylated/active Akt (Ser-473, Cell Signaling). (**b**) Western blot analysis using an antibody directed against PTEN (Cell Signaling) showing the expression of this lipid phosphatase in the prostate cell lines used in this study. (**c** and **d**) Western blot analysis shows time-dependent phosphorylation of Akt and GSK3β (Ser-9, Cell Signaling) induced by PDGF BB (30 ng/ml) in PC3 ML and PC3-N prostate cancer cell lines in the absence of serum. Cells collected while growing in serum, which underwent no experimental manipulation, are indicated as untouched (Unt), whereas cells deprived of serum for 4 h are indicated as starved (St). (**e** and **f**) Time-dependent phosphorylation of Akt induced by PDGF in PC-3ML and PC3-N cells in the presence of serum as shown by Western blot analysis. Experiments were performed on cell lines 3 days after plating and at approximately 80% confluence. For the experiments conducted in the absence of serum, serum-containing medium was removed, cells were washed twice with phosphate-buffered saline solution and then incubated with serum-free medium for 4 h. For the addition of growth factors, 20% of culture medium volume was removed, mixed with the growth factor, returned to each culture vessel and incubated at 37°C. At the times indicated, cells were lysed and SDS–PAGE and Western blot analysis was performed as described previously (Shulby *et al*., 2004), with few modifications. Samples from coupled experiments were run in the same gel to achieve a reliable comparison of band density. Blotted membranes were blocked and incubated with primary antibodies using NAP blocker (Genotech). Primary antibody binding was detected using HRP-conjugated Protein A or Protein G (both from Sigma). In some cases, biotinylated molecular weight markers were included in the gel. Chemiluminescent signals were obtained using SuperSignal West Femto reagents (Pierce) and detected with the ChemiDoc imaging system and relative software (Biorad Hercules). Densitometry analysis was performed using the UN-SCAN IT software (Silk Scientific). Antibodies against total Akt (Cell Signaling) or actin (Sigma) were used after membrane stripping to confirm uniform protein loading. Histograms represent band densitometry $\pm$ s.e. and were obtained from three separate experiments. Statistical significance was determined with the one-way ANOVA test (* = $P<0.005$)

2004). However, 72 h after injection only the PC3-ML cells could still be detected in the bone (Wang and Stearns, 1991), thus suggesting that the ability to survive in the bone environment might rather explain the different metastatic potential of these two subpopulations of prostate cancer cells. We therefore focused our attention on the PI3K/Akt signaling pathway, which is essential for the survival of tumor cells in general, and

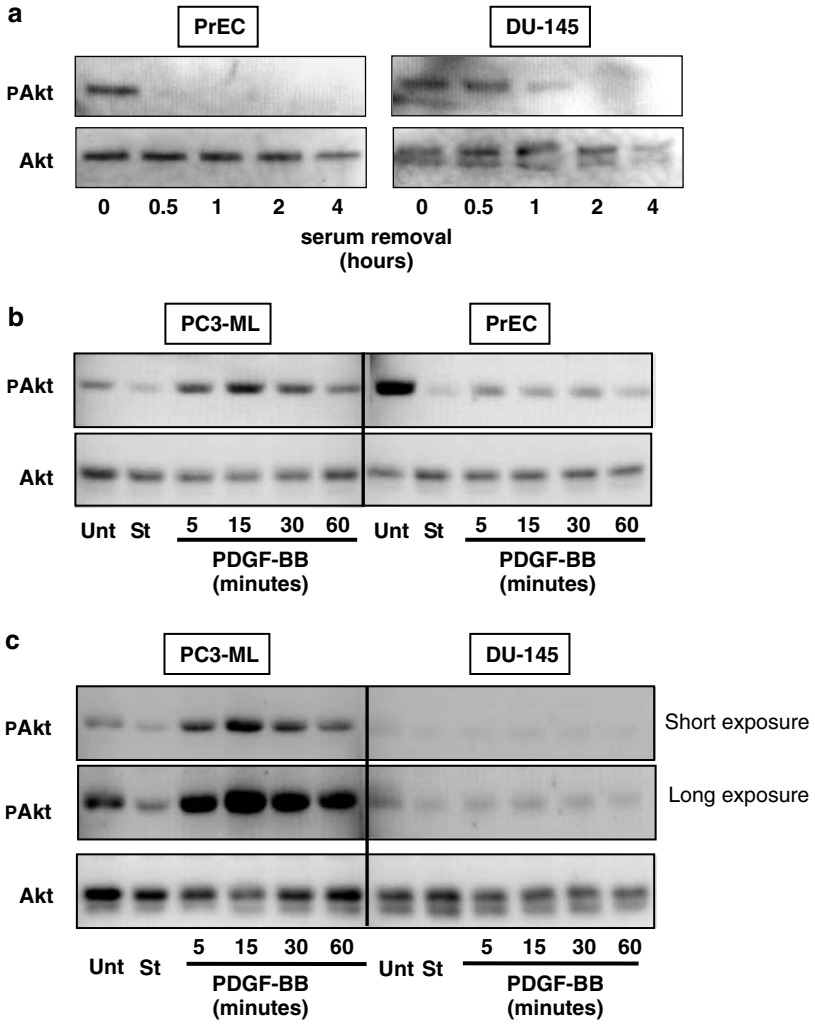


Figure 2 Normal PrEC display a normal phenotype and have a finite lifespan *in vitro* (Cambrex Bio Science). DU-145 cells are a human prostate carcinoma cell line of epithelial origin isolated from a brain metastasis (Stone *et al*., 1978). (**a**) Western blot analysis of time-dependent decrease in Akt phosphorylation (Ser-473) following serum deprivation in PrEC normal prostate epithelial cells and DU-145 cells. Serum deprivation induced a fast Akt dephosphorylation of both these cells types, which express PTEN (Figure 1b). (**b** and **c**) Exposure to PDGF-BB (30 ng/ml) failed to activate Akt as shown by Western blot analysis. Samples from either PrEC or DU-145 cells were run in tandem in the same gel with samples from PC3-ML cells to compare PDGF-induced effects on Akt phosphorylation. The overexposure of the membranes blotted with DU-145 samples show a very faint pAkt signal that was not affected by PDGF-BB (Long exposure). The levels of total Akt obtained after membrane stripping were used to confirm uniform protein loading. Each blot is representative of three separate experiments, which provided similar results

PCa cells in particular (Graff, 2002; Chang *et al*., 2003), and analysed the activation of the Akt kinase in PC3-ML and PC3-N cells upon PDGF exposure.

An initial series of experiments was conducted using PC3-ML and PC3-N cells deprived of serum for 4 h prior to exposure to 30 ng/ml PDGF-BB. This time point was chosen by monitoring the levels of time-dependent Akt dephosphorylation following serum removal. After 4 h of serum deprivation, some phosphorylated Akt was still detected, as these cells lack the lipid phosphatase PTEN, which negatively regulates Akt activation (Figure 1b). However, serum removal did significantly reduce the fraction of phosphorylated Akt compared to cells growing in serum-containing medium (Figure 1a), thus allowing the isolation of any PDGF-induced phosphorylation of the kinase. The PDGF-BB isoform was used, as it stimulates any combination of α- and β-PDGF receptors (PDGFR) (Heldin and Westermark, 1999), and we analysed the phosphorylation of the Ser-473 residue on Akt, an event necessary for the full activation of this kinase (Datta *et al*., 1999). Although both PC3-ML and PC3-N cells responded to the growth factor in a time-dependent manner, the magnitude of the Akt phosphorylation observed in PC3-ML cells was 3–4 times higher than that observed in PC3-N cells (Figure 1c and d). Phosphorylation of the Akt kinase at the Thr-308 residue was also induced by PDGF in both cell sublines and mirrored in magnitude and kinetic that observed at the Ser-473 level (not shown). This large difference in responsiveness to

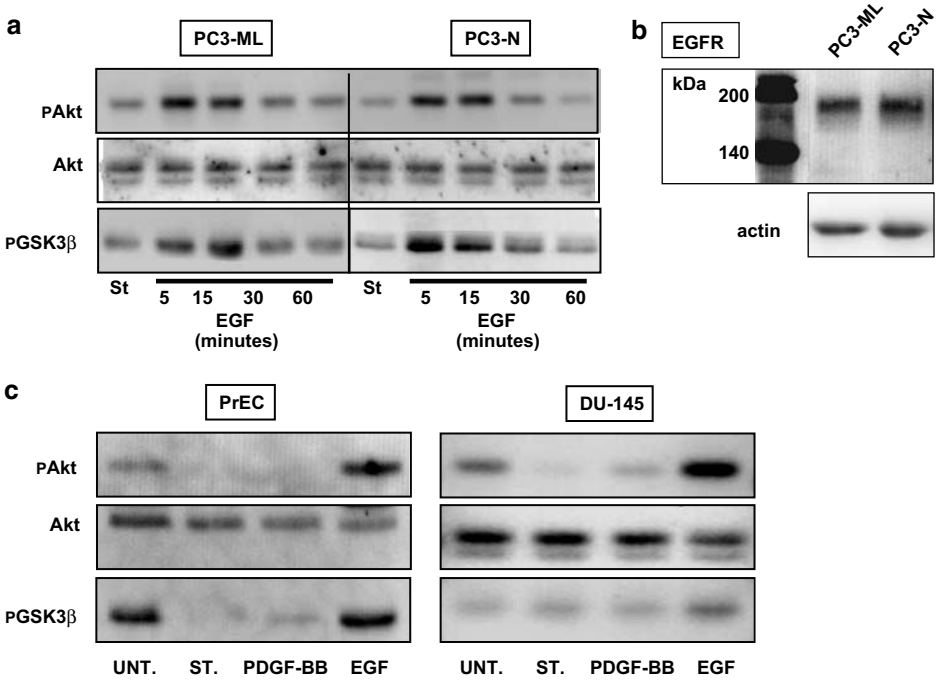


Figure 3 Western blotting analysis showing the effect of EGF (20 ng/ml) exposure on Akt and GSK3β phosphorylation in different prostate cell types. (**a**) PC3-ML and PC3-N cells responded to the growth factor in an identical manner, as shown by samples from coupled experiments run in the same gel to achieve a reliable comparison of band density. (**b**) Western blotting using an antibody directed against EGFR (Cell Signaling) shows that these two PC3 sublines express equal levels of the receptor. (**c**) PrEC and DU-145 cells also responded to EGF treatment for 15 min with Akt and GSK3β phosphorylation. Samples from PrEC and DU-145 cells treated with PDGF (30 ng/ml) for the same time length were included for comparison. Cell types were exposed to growth factors after being deprived of serum for 4 h. Cells not exposed to PDGF but collected while growing in serum are indicated as untouched (Unt), whereas serum-deprived cells are indicated as starved (St). The levels of total Akt or actin, obtained after membrane stripping, were used to confirm uniform protein loading. Each blot is representative of three separate experiments, which provided similar results

PDGF between PC3-ML and PC3-N cells was also evident when we investigated the phosphorylation of the Ser-9 residue of the downstream substrate glycogen synthase kinase-3β (GSK3β), which demonstrates the effective induction of Akt kinase activity (Datta *et al*., 1999). Interestingly, the phosphorylation levels of Akt and GSK3β induced by PDGF in serum-deprived PC3-ML cells were several folds higher than those observed in the same cells growing in the presence of serum. In contrast, PC3-N cells were characterized by PDGF-induced Akt and GSK3β phosphorylation only slightly higher than untouched controls.

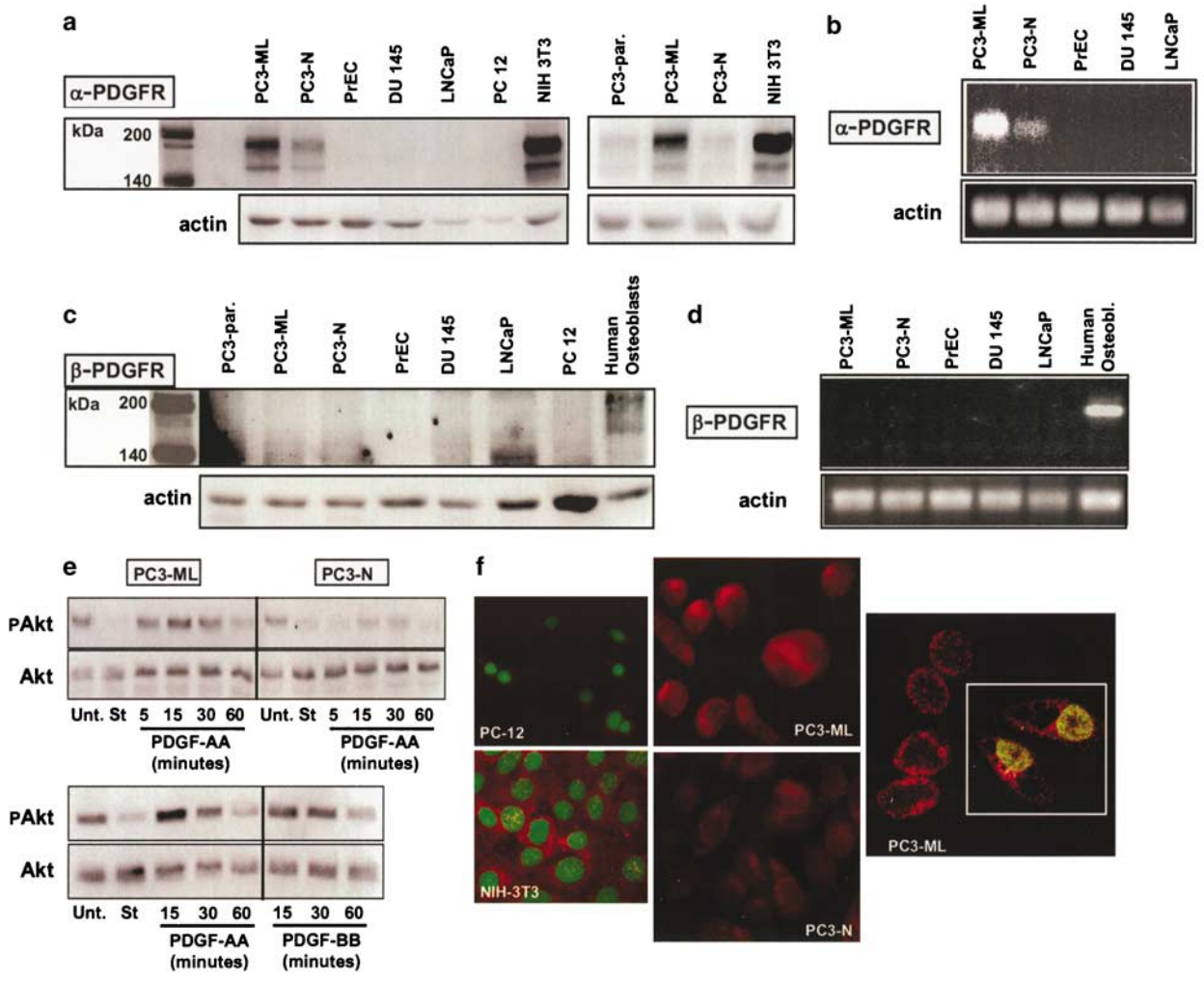
The *in vitro* exposure of cultured cells to receptor agonists in the absence of extracellular serum is a widely adopted procedure, which permits the identification of intracellular signals generated by the agonist/receptor of interest independently of any other receptor/pathway. However, this approach does not provide information about the relevance of receptors/pathways functioning in cells simultaneously exposed to physiological concentrations of other growth factors and hormones, as normally occurs *in vivo*. For instance, to be instrumental in the survival of prostate cancer cells in the bone marrow, PDGF should activate the Akt signaling pathway significantly above the level induced by other survival/trophic factors to which prostate cells spreading to different tissues are commonly exposed. Thus, in the next series of experiments, we investigated the activation of Akt induced in metastatic and nonmetastatic prostate cells by PDGF in the presence of 10% serum. The results we obtained indicate that PDGF activates the Akt signaling pathway also in these conditions and that the metastatic PC3-ML cells are significantly more responsive to its action than the nonmetastatic PC3-N cells (Figure 1e and f). As previously reported by others for the PC3 parental line and demonstrated in this study by us for the PC3-ML and PC3-N sublines (Figure 1b), these cells do not express PTEN, a lipid phosphatase that downregulates the PI3K-Akt pathway and has been proposed to be causally involved in prostate cell transformation (Majumder *et al*., 2003). However, our data show that in these two PC3 sublines the Akt pathway is not *maximally* upregulated by the lack of PTEN as they are able to further phosphorylate Akt in response to PDGF even in the presence of other serum-derived growth factors. Further mechanisms are likely responsible for modulating Akt activation and the undeniable tumorigenic contribution of PTEN loss might not be exclusively related to its modulation of Akt phosphorylation levels (Raftopoulou *et al*., 2004). It is also significant that despite PC3-ML and PC3-N cells lacking PTEN, their invasiveness *in vitro* and metastatic potential when injected in SCID mice are dramatically different (Wang and Stearns, 1991), suggesting that

deletion of this lipid phosphatase does not exclusively determine the invasive and metastatic phenotype of these two prostate sublines.

We were then interested in comparing the PDGF responsiveness of bone-metastatic PC3-ML cells to that of normal prostate epithelial cell (PrEC). When these cells were deprived of serum, they underwent a significantly faster dephosphorylation of Akt compared to both PC3 sublines (Figure 2a), likely because they express a functional PTEN (Figure 1b). However, the exposure of PrEC serum deprived for 4 h to PDGF did not induce any activation of the Akt signaling pathway (Figure 2b). Identical results were obtained using PrEC cells obtained from three different human donors (Cambrex Biosciences). Interestingly, in the presence of serum the basal levels of Akt phosphorylation observed in PrEC, which express PTEN, were significantly higher than those observed in PC3-ML cells, which do not express the lipid phosphatase (Figure 2b). This observation further underscores the relevance of mechanisms additional to PTEN involved in the control of Akt activity, such as the PP1 phosphatase (Xu *et al*., 2003).

The idea for a role of PDGF in determining the tissue tropism of prostate cancer cells inherently implies that prostate cells that are unable to take advantage of a PDGF-enriched microenvironment, such as the bone marrow, are more likely to develop metastases in other organs, which are better suited to sustain their survival. In fact, phenotypes with different growth factor sensitivities might very well emerge within the primitive tumor, a concept supported by the largely established cellular heterogeneity, which is a characteristic of prostate adenocarcinoma (Ruijter *et al*., 1999). To test this hypothesis, we performed the next experiments using DU-145, a prostate cell line originally derived from a brain metastasis. As previously observed for PrEC, the exposure of serum-deprived DU-145 to PDGF was unable to activate the Akt signaling pathway (Figure 2c). Thus, despite their malignant and metastatic phenotype, DU-145 cells fail to recruit Akt-dependent signaling in response to PDGF similarly to normal PrEC.

Epidermal growth factor (EGF) is normally found in prostatic fluid, is involved in the growth of human prostate and its increased expression has been linked to prostate cancer development (Russell *et al*., 1998). Analogously to PDGF, EGF binds and activates plasma membrane tyrosine kinase receptors (EGFRs) that transmit signals to the intracellular environment by



recruiting downstream effectors, including Akt. We therefore examined whether the discrepancies observed exposing different normal and malignant prostate cells to PDGF would be reproduced by EGF treatment. In these experiments, 20 ng/ml EGF induced phosphorylation of Akt and GSK3β with comparable efficacy in PC3-N and PC3-ML cells (Figure 3a). In line with this observation, the expression levels of EGFR were similar in PC3-ML and PC3-N cells (Figure 3b) as well as in PrEC, DU-145 and LNCaP cells, which were originally derived from a lymph node metastasis (not shown). In addition, the nonmalignant PrEC cells exposed to EGF also showed a strong Akt activation, similarly to the brain-derived DU-145 cells (Figure 3c). Thus, prostate cells exposed to EGF activate Akt in a similar manner independently from their phenotype or metastatic tissue of origin, suggesting that the recruitment of the Akt signaling pathway by EGFRs would be less relevant, if at all, in determining the bone tropism of disseminated prostate cancer cells.

To identify the mechanism responsible for such a wide discrepancy in PDGF-induced stimulation of the Akt survival pathway among normal and malignant prostate cells, we investigated the expression levels of both α- and β-PDGFR in each cell type used for this study and also in LNCaP cells. As shown in Figure 4a (left panel), Western blotting analysis detected the α-PDGFR isoform exclusively in PC3-ML and PC3-N sublines and, as expected, in the parental PC3 line (Figure 4a, right panel). Most importantly, the expression levels of α-PDGFR in PC3-ML cells were significantly higher than those observed in PC3-N cells. These results were also confirmed by Northern blotting analysis (Figure 4b). It should also be noted that α-PDGFR expression levels in the parental PC3 cells and PC3-N sublines were comparable (Figure 4a). This could be explained by the fact that less than 1% of the parental PC3 cells were initially selected for their invasiveness *in vitro* and then used to originate the PC3-ML subline. Thus, the parental PC3 cell population can be considered represented almost entirely by PC3-N cells. In agreement, parental PC3 and PC3-N cells stimulated with PDGF activated the Akt pathway to a similar magnitude (not shown). When examined for β-PDGFR expression, all the prostate cells tested negative to both Western and Northern blotting analyses (Figure 4c and d). These results were further confirmed by the exposure of PC3-ML and PC3-N cells to PDGF-AA, which stimulates the α-PDGFR but not the β-isoform (Heldin and Westermark, 1999) and induced Akt phosphorylation in a manner similar to that previously shown for PDGF-BB (Figure 4e). Analogously, the exposure of PC3-ML cells to either PDGF-AA or PDGF-BB determined an increase in Akt phosphorylation of comparable magnitude, thus conclusively excluding the additional stimulation of β-PDGFR by PDGF-BB (Figure 4e). Finally,

Figure 4 (**a**) The expression of α-PDGFR by the prostate cells used in this study was analysed by Western blotting using three different antibodies obtained from Cell Signaling (3164), R&D Systems (AF-307-NA) and Santa Cruz (sc-338), which provided identical results. Samples from PC-12 cells were included as a negative control and samples from NIH 3T3 fibroblasts as a positive control. (**c**) Western blotting analysis using three antibodies directed against the β-PDGFR peptide from R&D Systems (AF385), Cell Signaling (3162) and Santa Cruz (sc-339), which produced identical results, showed all prostate cell lines to lack expression of the receptor β-isoform. Human osteoblasts and PC-12 cells were included as positive and negative controls, respectively. Membranes were stripped and probed for actin levels to confirm uniform protein loading. Northern blotting was performed to detect levels of α-PDGFR (**b**) and β-PDGFR (**d**) messenger RNA levels, which corresponded to the protein expression profiles produced by Western blotting. Duplicate membranes were probed for actin to verify equal RNA loading. Total RNA was isolated using the RNeasy Mini kit (Qiagen) according to the manufacturer's protocol. Total RNA (10 μg) was separated through a 1.2% agarose/formaldehyde gel and transferred to GeneScreen Plus nylon membrane (Perkin-Elmer). After transfer, the RNA was fixed to the membrane by UV crosslinking. Equal loading and integrity of the RNA was evaluated by methylene blue staining of the membrane. The membranes were then hybridized at 65°C to random primed ^{32}P-labeled probes. Probes were generated using the Radprime kit (Invitrogen) according to the manufacturer's protocol. Probes correspond to the following sequences: α-PDGFR (NM_006296.2): nt 960–2230; β-PDGFR (BCO3224): nt 3918–5216; and β-actin (NM_001101): nt 685–1171. After hybridization and washing, the membranes were visualized and quantitated by phosphorimage analysis (Amersham Biosciences). (**e**) PC3-ML and PC3-N cells exposed to 20 ng/ml of PDGF-AA respond with a pattern of Akt phosphorylation similar to that induced by PDGF-BB (top panel), while the exposure of PC3-ML cells to either PDGF-AA or PDGF-BB increased Akt phosphorylation with a comparable magnitude (bottom panel). (**f**) The expression of α-PDGFR was also characterized by immunofluorescence using an antibody first tested for the absence of staining in α-PDGFR-negative PC-12 cells and strong red fluorescent signal in positive NIH 3T3 cells (left panels). Conventional immunofluorescence confirmed the higher α-PDGFR expression in PC3-ML compared to PC3-N cells (center panels). The presence of α-PDGFR at the plasma membrane level of PC3-ML cells was detected using confocal microscopy and indicated by the ring-like appearance of the fluorescent signal (right panel). Nuclei in left and right panels were stained in yellow–green using SYTOX Green (Molecular Probes). For conventional immunofluorescence, cells were grown on 15 mm glass coverslips and fixed with 4% paraformaldehyde. Primary antibodies against the α-PDGFR obtained from different commercial sources were validated for immunofluorescence by using PC12 cells, which lack the α-PDGFR and NIH 3T3 cells, which express high levels of the receptor, as negative and positive controls, respectively. The antibodies that did not provide any signal in PC12 cells while strongly staining the NIH 3T3 cells were used to detect the α-PDGFR in the PC3 sublines. According to these criteria, an antibody against the extracellular domain of human α-PDGFR obtained from R&D Systems (AF-307-NA) was used on nonpermeabilized cells to impede its access to the intracellular environment. The primary antibody was followed by a biotinylated secondary antibody and Cy3-conjugated streptavidin (both from Jackson ImmunoResearch Labs). Fluorescent images were acquired using a CCD MicroMax 1300YHS digital camera (Roper Scientific) connected to an IX-70 microscope (Olympus) and managed by a computer equipped with the Metamorph software (Universal Imaging Corporation). For confocal analysis cells were grown on 15 mm glass coverslips and fixed with a pH-shift paraformaldehyde protocol, to avoid cell shrinkage as well as maintaining the spatial cellular organization. An antibody against human α-PDGFR from Cell Signaling (3146) was employed for confocal microscopy analysis. Samples were imaged using a Nikon Diaphot 300 inverted microscope mounting a 60×1.40NA oil-immersion objective. Images were collected using a step size of 0.5 μm along the z-axis and at a resolution of 1280×1280 using the C-Imaging software

the difference in α-PDGFR expression between PC3-ML and PC3-N cells was uniformly observed within each cell population, as demonstrated by immunofluorescence detection (Figure 4f). This rules out the possibility that the PC3-ML subline is composed by only a limited fraction of cells expressing very high levels of the receptor, which could not be conclusively excluded by Western blotting analysis. The location of the α-PDGFR at the plasma membrane level was confirmed by confocal microscopy analysis (Figure 4f).

In conclusion, we investigated the correlation between growth factor responsiveness and bone-metastatic potential in prostate cancer sublines derived from the same parental population. This approach allowed us to significantly restrict the number of possible differences in phenotype commonly observed among cancer cells derived from separate individuals. A crucial observation emerging from our study is that PC3-ML cells, which were originally selected for their high bone-metastatic potential, express significantly higher levels of α-PDGFR compared to malignant but nonmetastatic PC3-N cells and activate the Akt pathway more effectively when exposed to PDGF. The differences in the expression and signaling of α-PDGFR are not reproduced when these two cell types are tested for the EGFR, thus hinting to a unique correlation between α-PDGFR upregulation and functioning in bone-metastatic prostate cells. Furthermore, these findings seem to confer a more determinant role to PDGF in regulating the survival of bone-metastatic prostate cells compared to EGF. Indeed, this hypothesis is supported by the fact that within the skeleton, the bone marrow contains significant levels of PDGF, whereas the expression of EGF is confined to the trabeculae (Watson *et al*., 1996). Thus, the different susceptibility to the trophic action exerted by PDGF could at least in part explain why both PC3-ML and PC3-N cells locate to the skeleton of SCID mice with comparable efficiency after their injection in the blood stream but only the PC3-ML cells are able to grow into bone metastases (Wang and Stearns, 1991). Finally, it would be intriguing to speculate that prostate cells originally derived from brain (DU-145) or lymph node (LNCaP) metastases, which express EGFRs but lack α-PDGFR signaling, represent selected tumor phenotypes unfit to respond to PDGF-induced survival signals and therefore unable to support their growth in bone tissue.

Immunohistochemical analysis of PDGFR expression in prostate tissue has previously shown that normal epithelium stains negative to both α- and β-isoforms, in line with our results obtained with PrEC, whereas adenocarcinoma shows an evident staining for the α-PDGFR (Wang and Stearns, 1991). The apparent discrepancy that DU-145 and LNCaP cells, despite their malignant phenotype, do not express α-PDGFRs could be related to the characteristic histopathological heterogeneity of PCa. In turn, the expression of α-PDGFR would indeed represent an indication of malignant transformation, but it might not be extended throughout the entire cancer cell population in the prostate gland. In fact, our study shows that the expression of α-PDGFR could rather identify those cells within the primitive tumor, which possess the highest propensity to metastasize to the skeleton.

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