Human prostate cancer harbors the stem cell properties of bone marrow mesenchymal stem cells.

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**Running Title:** Stem cell properties of human prostate cancer.
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Statement of translational relevance

This work is the first to define the lineage-specificity of prostate cancer stem cells. It can be applied to the future practice of cancer medicine, since several salient features of the work warrant further investigative research and therapeutic development. First, bone marrow mesenchymal stromal markers can be used in prostate cancer detection and diagnosis, since these markers are abnormally expressed in prostate cancer cells, and since the expression level is correlated to the disease progression. Second, the bone marrow mesenchymal stem cell properties of prostate cancer will be further scrutinized as an inherent cause of the preferred bone metastasis. Such studies will lead to development of stem cell therapy that can be broadly exploited to prevent prostate cancer bone metastasis. Finally, because prostate cancer cells can be induced to differentiate in a lineage-specific manner, therapeutic strategies can be developed by pharmaceutically inducing prostate cancer cells to differentiate. As demonstrated in this study as well as reported by other investigators, differentiation therapy leads to growth arrest and apoptotic death of prostate cancer cells.
Abstract

**Purpose:** Prostate tumor cells frequently show the features of osteoblasts, which are differentiated from bone marrow mesenchymal stem cells. We examined human prostate cancer cell lines and clinical prostate cancer specimens for additional bone marrow mesenchymal stem cell properties.

**Experimental Design:** Prostate cancer cell lines were induced for osteoblastogenic and adipogenic differentiation, detected by standard staining methods and confirmed by lineage-specific marker expression. Abnormal expression of the markers was then assessed in clinical prostate cancer specimens.

**Results:** After osteoblastogenic induction, cells of the LNCaP lineage, PC-3 lineage and DU145 displayed osteoblastic features. Upon adipogenic induction, PC-3 lineage and DU145 cells differentiated into adipocyte-like cells. The adipocyte-like cancer cells expressed brown adipocyte-specific markers, suggesting differentiation along the brown adipocyte lineage. The adipogenic differentiation was accompanied by growth inhibition, and most of the adipocyte-like cancer cells were committed to apoptotic death. During cyclic treatments with adipogenic differentiation medium and then with control medium, the cancer cells could commit to repeated adipogenic differentiation and retrodifferentiation. In clinical prostate cancer specimens, the expression of UCP1, a brown fat-specific marker, was enhanced with the level of expression correlated to disease progression from primary to bone metastatic cancers.

**Conclusions:** This study thus revealed that prostate cancer cells harbor the stem cell properties of bone marrow mesenchymal stem cells. The abnormally expressed adipogenic UCP1 protein
may serve as a unique marker, while adipogenic induction can be explored as a differentiation therapy for prostate cancer progression and bone metastasis.
Introduction

Stem cells have two unique properties, the ability to undergo self-renewal and the potential to differentiate into lineaged mature cells (1). Prostate cancer cells have stem cell-like properties, since cancer cells undergo incessant cell division in a fashion similar to the self-renewal of stem cells (2, 3), and produce heterogeneous progeny (4, 5). Supporting studies demonstrated that prostate cancer specimens display sporadic expression of stem cell markers (6, 7), and tumor cells enriched with these markers were shown to have increased tumorigenic potential (7, 8). Prostate tumors are thought to have a hierarchical organization, with a small number of cancer-initiating stem cells undergoing self-renewal and producing a heterogeneous tumor cell population (9, 10). Because stem cells are mostly insensitive to conventional therapy, cancer stem cells may account for tumor recurrence. Defining the stem cell properties of the tumor cells may facilitate the development of effective therapies.

Prostate tumor cells frequently express features of the bone marrow mesenchymal stem cell lineage. Bone-specific markers, for instance, are normally expressed in osteoblasts, which are differentiated from the multipotent bone marrow mesenchymal stem cells. Prostate tumors, however, abnormally express Cbfa1/RUNX2, the essential transcription factor for osteoblastic differentiation (11), whereas osteomimetic expression of bone-specific markers such as osteocalcin, osteopontin, and bone sialoprotein is correlated to the bone metastatic potential of the tumor (12, 13). In addition, prostate cancer cells can be induced to show osteoblastic mineralization (14). These findings suggest that prostate cancer cells have the stem cell properties of bone marrow mesenchymal stem cells.
Mesenchymal stem cells are pluripotent. Besides differentiating into mature osteoblasts, bone marrow mesenchymal stem cells may differentiate to other mature cell types such as adipocytes, depending on induction cues (15). We reasoned that the bone marrow mesenchymal stem cell properties of prostate cancer cells may be assayed under similar conditions. In this report, we examined commonly used human prostate cancer cell lines to investigate whether these cells harbor the properties of bone marrow mesenchymal stem cells, by inducing the cells for osteoblastic and adipogenic differentiation. Results from this study revealed that prostate cancer cell lines had the potential to differentiate into osteoblast-like cells, while some could additionally differentiate into adipocyte-like cells. Adipogenic marker was detected abnormally expressed predominantly in clinical prostate cancer specimens. Importantly, the adipogenic differentiation resulted in growth inhibition and death of the adipocyte-like cancer cells. This study thus exposed stem cell properties in prostate cancer cells and the lineage-specificity of prostate cancer stem cell differentiation.

**Experimental Procedures**

*Reagents.* Cell culture grade glucose, dexamethasone, β-glycerolphosphate, L-ascorbic acid 2-phosphate, L-thyroxine, insulin, and indomethacin were purchased from Sigma-Aldrich (St. Louis, MO). Alpha Minimum Essential Medium (αMEM), Keratinocyte Serum Free Medium (K-SFM), bovine pituitary extract (BPE), human recombinant epidermal growth factor (EGF), and glutamine were from Invitrogen (Carlsbad, CA). Alizarin Red S and Oil Red O were from Sigma-Aldrich.
Cell lines and cell culture conditions. The sources and culture of human prostate cancer cell lines LNCaP, C4-2, C4-2B, PC-3, PC-3M, and DU145 were reported previously (16). The RWPE-1 immortalized human prostate epithelial cell line was purchased from American Type Culture Collection (Manassas, VA) and propagated in K-SFM containing BPE (0.05 mg/ml) and EGF (5 ng/ml). Normal human bone marrow mesenchymal stromal cells (hMSC) and mouse bone marrow mesenchymal stromal cells (mMSC) were purchased from the Tulane Center for Gene Therapy (Tulane University, New Orleans, LA), and were cultured in αMEM containing 16.5% fetal bovine serum (FBS) and 2 mM L-glutamine.

Osteoblastic and adipogenic induction. To assay for osteoblastic differentiation, cells at 70% confluence were treated for 21 days in osteoblastic differentiation medium, which was αMEM containing FBS (5%), glucose (4.5 mg/ml), 4 mM glutamine, 1 nM dexamethasone, 20 mM β-glycerolphosphate, 50 μM L-ascorbic acid 2-phosphate, and L-thyroxine (50 ng/ml). Adipogenic differentiation was induced by treating cells for 21 days in adipogenic differentiation medium, which was αMEM containing FBS (5%), glucose (4.5 mg/ml), 4 mM glutamine, insulin (5 μg/ml), 100 nM dexamethasone, and 50 μM indomethacin. Cells in the control group were treated for 21 days with control medium, which was αMEM containing FBS (5%), glucose (4.5 mg/ml), and 4 mM glutamine.

Cell staining. After removal of the culture medium, cells were rinsed gently with phosphate buffered saline and fixed in 10% formalin for 20 minutes. For Alizarin Red S staining (14), fixed cells were incubated in 1% Alizarin Red S, pH 4.3, for 20 minutes. For Oil Red O staining
fixed cells were treated with 0.3% Oil Red O in 60% isopropyl alcohol for 20 minutes. After removal of free dyes by washing, cells were subjected to microscopic documentation.

**Proliferation assay.** The protocol used for assaying cell proliferation with MTT conversion was reported previously (18). In this study, equal numbers of cells (5 x 10^5/ml) were plated onto a 96-well plate. After treatment under differentiation conditions for 7 days, the cells were subjected to a proliferation assay.

**Fluorescence-activated cell sorting (FACS) analysis.** The protocol used for assaying apoptotic cells with FACS was reported previously (19). In this study, cells treated under differentiation conditions for 14 days were collected and subjected to FACS analysis.

**DNA fragmentation assay.** The protocol used for assaying fragmented genomic DNA was reported previously (20). In this study, cells treated under differentiation conditions for 14 days were collected and subjected to DNA fragmentation assay.

**RT-PCR.** The protocol used for RT-PCR analysis of gene expression was reported previously (16). Oligonucleotide primer pairs used in the study were 5’-

ATGGGTGAAACTCTGGGAGATTCTCCTATTGAC-3’ and 5’-

CAAACCTGATGGCATTATGAGACATCCCCACTG-3’ for the peroxisome proliferator-activated receptor γ (PPARγ; GenBank accession number NM_015869), 5’-

ATGTGTGATGCTTTTGTAGGTACCTG-3’ and 5’-

TTATGCTCTTCATAAACTCTCGTGGAAGTGAC-3’ for fatty acid binding protein 4
(FABP4/aP2; NM_001442), 5’-CCCTTGGGAAGGATTGGACCCATCAGGGCATC-3’ and 5’-TTACGCTGAGGCAGAATGAATGTCCTG-3’ for lipin 1 (LPIN1; NM_145693), and 5’-TGATGAGAAGTGTCACTCATCAATTGTACAGAG-3’ and 5’-TTATGTGGCACAGTCCATAGTCTGCTTGACTTTG-3’ for uncoupling protein 1 (UCP1; NM_021833). Primer pairs used to detect the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were previously reported (19).

**Immunohistochemical (IHC) assays.** Surgically removed human prostates were examined with IHC for specific gene expression, using human prostate cancer specimens as approved by the local institutional review board. The source of one normal prostate and two primary prostate tumor specimens was previously reported (19). In addition, two sets of tissue microarray (TMA) were subjected to IHC assay in this study. The constitution of the first TMA has been described previously (19), with samples from 32 prostates, including 4 healthy prostates and 2 with benign prostatic hyperplasia (BPH). Prostates with histopathologically confirmed cancer were represented by triplicate samples. The TMA contained matched specimens of benign, prostatic intraepithelial neoplasia (PIN) and tumor tissues from 12 prostates; matched benign and tumor tissues from 11 prostates; matched PIN and tumor tissues from 1 prostate; and specimens of prostate cancer bone metastasis from 2 patients. The second TMA consisted of triplicate bone metastasis tissue cores from 15 prostate cancer patients who died from metastatic prostate cancer, accrued under the aegis of the Prostate Cancer Donor Program at the University of Washington Medical Center (21).
To determine androgen receptor (AR) status, a TMA was subjected to IHC detection with F39.4.1 monoclonal antibody (Biogenex Laboratories, Fremont, CA) at 1:60 dilution. An indirect avidin-biotin immunoperoxidase method was used, with biotylated secondary antibodies and a diaminobenzidine detection system (Vector Laboratories, Burlingame, CA). Polyclonal antibodies to human UCP1 were purchased from Abcam (ab10983, Cambridge, MA) and used as primary antibodies at 1:500 dilution. IHC staining was conducted with the Dako Autostainer Plus system (Dako Corporation, Carpinteria, CA), using a previously reported protocol (19).

The method used to score IHC results was previously described (19), with specific stains graded from 0 (negative) to 4 (intensely positive). AR status was determined by tabulating cytoplasmic and nuclear stains separately. To quantify and compare the level of UCP1 expression between prostate cancer specimens, stain intensity was combined with the extent of positive cells over the tumor area, which was assigned incrementally from 1 (less than 20% of tumor cells were stained) to 4 (more than 60% of tumor cells were stained). The signal intensity and extent scores were added together to reflect UCP1 expression in each specimen.

Statistical analysis. Student’s t-test was used in statistical comparison between unpaired groups. Level of statistical significance between control and treated groups was set at $P < 0.05$.

Results

1. Bone marrow mesenchymal stem cell properties in prostate cancer cell lines.
Prostate cancer cells were treated for 21 days respectively with osteoblastic differentiation medium and adipogenic differentiation medium, following established protocols that were used to induce differentiations of bone marrow mesenchymal stem cells (15). Normal human bone marrow mesenchymal stem cells, hMSC, were used as a positive control. Subsequently, standard methods were used to detect osteoblastic differentiation with Alizarin Red S (14). Adipogenic differentiation was detected with a standard staining method using Oil Red O (17).

In the control group where cells were not treated for osteoblastic induction, there were higher background stains in prostate cancer cell lines than in normal prostate epithelial cells (Figure 1A). This was in agreement with previous reports that prostate cancer cells, even under conventional culture conditions, showed features mimicking osteoblastic cells (12, 13). We found that after osteoblastic induction, prostate cancer cells produced more intense Alizarin Red S stains than the untreated cells in general (Figure 1A). The staining was especially profound in cells of the LNCaP lineage (LNCaP, C4-2, and C4-2B) and the PC-3 lineage (PC-3 and PC-3M). In contrast to the uniform intracellular stains seen in the LNCaP and PC-3 lineages, stains in the DU145 prostate cancer cells appeared mostly in large clusters covering large areas of cells, suggestive of extracellular matrix mineralization. Examined by RT-PCR analysis in LNCaP lineaged cells, the expression of osteoblastic markers osteocalcin and osteopontin was increased 8 days after osteoblastic induction, while bone sialoprotein was induced between 8 and 16 days. Importantly, RUNX2/Cbfa1, the master transcription factor of osteoblastogenesis (11), was also induced. The expression pattern of osteoblast markers was in agreement with the results previously reported (11-13). In control groups, Alizarin Red S staining in hMSC cells revealed frequent osteoblastic differentiation, while no specific staining was detected in the immortalized
normal human prostate epithelial RWPE-1 cells (Figure 1A). These results suggested that prostate cancer cell lines could be induced to differentiate into osteoblast-like cells.

Besides differentiating into osteoblasts, bone marrow mesenchymal stem cells can produce several other mature cells, including adipocytes. We examined whether prostate cancer cells have similar potential by assaying adipogenic differentiation. Prostate cancer cells under adipogenic induction produced more intense Oil Red O stains than the untreated cells, while different cancer cell lines again showed varied staining intensities (Figure 1B). Cells of the LNCaP lineage showed weak staining, whereas PC-3 lineage displayed prominent staining. Treated PC-3 and PC-3M cells contained multiple small lipid droplet-like organelles full of the cytoplasm, similar to the morphology of brown fat cells (22). The lipid droplet-like organelles in DU145 cells were even tinier. In comparison, adipocytes differentiated from the hMSC cells contained large lipid droplets similar to white fat cells (22). No treated RWPE-1 cells were detected with lipid droplets. These results indicated that PC-3 lineage and DU145 prostate cancer cells could be induced to differentiate into brown adipocyte-like cells.

2. Characteristics of prostate cancer cell adipogenesis.

Human prostate cancer cell lines express osteoblast-specific genes (13) and can be induced to produce osteoblastic mineralization (14), suggestive of an osteoblastic stem cell property. Indeed, clinical prostate cancer specimens were found to abnormally express osteoblast markers such as RUNX2/Cbfa1, osteocalcin, osteopontin, and bone sialoprotein (11-14, 23, 24). Prostate cancer osteomimicry has been studied as an adaptation mechanism for the preferential metastasis
to the bone. On the other hand, none of the prostate cancer cell lines has been shown to have the potential to differentiate along the adipogenic lineage. In this report, we focused our characterization of the adipogenic differentiation in prostate cancer cells.

To assess the kinetics of the adipogenesis, PC-3, PC-3M, and DU145 cells were treated in adipogenic differentiation medium, and were assayed weekly for adipogenic differentiation. At 7 days of induction, the appearance of lipid droplets was scarce in PC-3 and DU145 cells (Figure 2A), but was already pronounced in PC-3M cells. Adipogenic features became prominent in PC-3 and DU145 cells after 14 days of induction (Figure 2A), while at this time adipogenic differentiation in PC-3M cells had reached a plateau. At 21 days of induction, more than 95% of the adipocyte-like PC-3M cells had died, compared to the death of about 80% of the adipocyte-like PC-3 cells. The surviving cells adopted dramatically enlarged shape, with lipid droplet-like organelles full of cytoplasm (Figure 2A). At this time, DU145 cells were found full of tiny lipid droplets, but less than 20% of the cells had died. Further treatment for adipogenic differentiation resulted in the death of all the PC-3M cells at 28 days and all the PC-3 cells at 42 days, whereas majority of the DU145 cells remained alive at the end of a 42-day induction. It seemed that each of the prostate cancer cell lines followed an intrinsic differentiation program.

Adipocytes express specific genes for lipid formation and metabolism (15, 25). RT-PCR analysis was used to examine whether adipogenic markers were expressed in prostate cancer cells. We found that PPARγ, the master transcription regulator for adipogenic differentiation (25, 26), was highly expressed (Figure 2B). Similarly, FABP4/aP2 was constitutively expressed, while LPIN1 was seen at a lower level and was induced transiently around 4 days of the
induction (Figure 2B). Interestingly, the expression of UCP1, a brown adipocyte-specific gene functioning to uncouple energy metabolism (27), was induced 4 days into adipogenic induction (Figure 2B). These results thus revealed that prostate cancer cells could express adipocyte-specific markers both constitutively and after adipogenic induction. The induced expression of UCP1 indicated that the prostate cancer cells were differentiating into brown fat-like cells, which function mainly in catabolic energy consumption (22, 25).


Normal stem cells are sustained by self-renewal. Committing to differentiation, a stem cell would enter a transient amplifying phase to produce progeny, which would exit the cell cycle to become terminally differentiated (28). Mature cells have limited life spans and would perish eventually through programmed mechanisms (29).

There was a dramatic loss of PC-3 lineaged cells after adipogenic induction (Figures 1B and 2A). We observed that growth of the cancer cells became slowed once in the differentiation medium, while cell death occurred later, mainly in the second and third week, after most cells had adopted adipocyte-like morphology. In cell proliferation assays, adipogenic induction resulted in significant growth inhibition after at 7 days (Figure 3A). In cell death assays, significant cell death was detected after a 14-day induction (Figure 3B). The dead cells were found to contain fragmented genomic DNA (Figure 3C), indicating an apoptotic death. These results suggested that PC-3 lineaged cells responded to adipogenic induction with growth arrest and
differentiation. Under the induction, the differentiated adipocyte-like cancer cells would perish through apoptosis.

4. Retrodifferentiation of prostate cancer cells after adipogenic induction.

Differentiation in normal situations is mostly an irreversible process (30). In contrast, reversible differentiation, or retrodifferentiation, may be an intrinsic feature of cancer stem cells (31, 32). We assessed whether the adipogenic differentiation in prostate cancer cells was reversible. Cells of the PC-3 lineage were first treated with a 21-day adipogenic induction. At the end of the induction, the small number of surviving cells all showed adipocyte-like morphology (Figure 4). Control medium was used to replace adipogenic differentiation medium to rescue these cells. After the change of medium, there was a latent phase of about 4 days, in which no cell division was seen but a gradual loss of the adipocyte-like morphology. After the latency, the cells started to divide to form colonies, which became discernible 7 days into the rescue (Figure 4). After a 21-day culture, the adipocyte-like morphology was lost and all the cells from these colonies assumed a new morphology indistinguishable from the original cancer cells (Figure 4). Adipogenic differentiation in the surviving cancer cells was thus reversible, and differentiated prostate cancer cells had the potential for retrodifferentiation.

The rescued cells retained adipogenic differentiation potential, with both the rates of growth inhibition and death of the adipocyte-like cells resembling those of the original prostate cancer cell lines. In two separate experiments, both PC-3 and PC-3M cells were subjected to three consecutive cycles of adipogenic induction and rescue, and similar results were observed from
each adipogenic induction. Prostate cancer cells surviving adipogenic induction had the potential to undergo repeated differentiation and retrodifferentiation (Figure 4).

5. Abnormally enhanced adipogenic marker expression in prostate cancer specimens.

This study demonstrates that the commonly used prostate cancer cell lines all have adipogenic differentiation potential, albeit to varied degrees (Figures 1, 2, and 4). To investigate whether adipogenic differentiation is a feature shared by tumor cells in clinical prostate cancer progression and bone metastasis, we examined adipogenic marker expression in a total of 45 human prostates by IHC assays. Here we present our findings regarding UCP1, an adipogenic marker exclusive to brown fat cells (27) that we found to be induced in prostate cancer cells (Figure 2).

UCP1 expression in normal human prostates was assessed. Relative to control staining, the UCP1 signal is low but discernible in normal prostates and BPH specimens, with weak staining seen in the epithelial compartment. In most benign specimens from cancer-afflicted prostates, cells of the basal layer show more prominent staining than luminal cells in general (Figure 5A). In normal prostates UCP1 seems to be expressed at a low level, and in the benign regions of cancer-afflicted prostates, a low level of UCP1 staining is mainly found in certain cells in the basal layer.

In cancer-afflicted prostates, we compared UCP1 levels in matched samples for benign, PIN, and tumor from the same prostate in a TMA format. An additional TMA containing specimens of
prostate cancer bone metastases was examined. Relevant background information on these cancer cases is summarized in Table 1. Compared to the low level in normal prostates and in benign regions, expression of UCP1 in prostate tumors was enhanced. Between matched samples, stronger staining was seen in PIN lesions and cancer than in benign regions (Figure 5B). In the tumor area, clustered cancer cells of larger size were differentially stained over smaller cancer cells. In both well-differentiated and poorly differentiated primary tumors (Figure 5C), enhanced expression was especially prominent in larger tumor cells. The stains in these cells appeared to be granulated; the stain in the cytoplasmic region presented a grainy appearance under higher magnification. UCP1 is transcribed from a nuclear gene and the protein is integrated to the inner membrane of mitochondria. The stain pattern probably reflected the mitochondrial localization of the UCP1 protein. Among all the specimens examined, the strongest staining was in all the 17 bone metastatic specimens. In many cases, bone metastatic cancer cells had uniform staining, and cancer cells in the trabecular space were stained the strongest (Figure 5D). Similar to primary cancer cells, many positive tumor cells in the bone showed larger cell sizes, with intense UCP1 signals distributed in a granulated fashion (Figure 5E). Interestingly in many bone metastases, the bone tumor cells frequently had minute lipid droplet-like structures full of cytoplasm (Figure 5F), reminiscent of brown fat cell morphology. In the 15 bone metastasis cases that had additional clinical pathologic information (Table 1), levels of high UCP1 expression were not correlated to Gleason scores of the primary cancer at diagnosis or years of survival of the patients. A high level UCP1 was seen in both AR-positive and AR-negative tumors, suggesting that the abnormal adipogenic marker expression in prostate cancer cells was independent of the AR status. In all the bone metastasis specimens examined, the extent of the enhanced expression appeared mostly unanimous, with less deviation than the
erratic PSA levels measured in the same patients (Table 1). Based on these results, we concluded that expression of the adipogenic marker UCP1 was abnormally enhanced in prostate cancer, and the enhancement was correlated to malignant stage, but independent of hormonal status of the disease.

**Discussion**

While investigating prostate cancer stem cell properties, we found that commonly used human prostate cancer cell lines could be induced to differentiate into osteoblast-like (Figure 1A) or adipocyte-like cells (Figure 1B). These findings are in concordance with clinical observations, in which prostate tumor cells often express osteoblastic markers (12-14, 23, 24). The results are also in agreement with previous studies where LNCaP and C4-2 prostate cancer cells were induced to express osteoblastic phenotypes (14, 33). In this study, we additionally demonstrated that the tumor cells could express adipogenic markers as well. The brown adipocyte-specific UCP1, for example, was abnormally enhanced in most primary tumors and all bone metastases (Figure 5), suggesting that in clinical prostate cancer, certain tumor cells may harbor mesenchymal stem cell properties as well. Osteoblastic and adipogenic differentiation are unique capabilities of bone marrow mesenchymal stem cells. Human prostate cancer cells thus harbor the bone marrow mesenchymal stem cell properties. The bone marrow is a unique microenvironment for mesenchymal stem cells, and tumor cells with bone marrow mesenchymal stem cell-like properties could have an advantage for homing to and colonizing bone. It would be intriguing to investigate whether these intrinsic properties are an underlying cause of the preferential metastasis of prostate cancer to bone.
This study showed that different prostate cancer cell lines have distinct potentials for lineage-specific differentiation. Cells of the LNCaP lineage appeared to have the potential to differentiate into osteoblast-like cells, while adipogenic differentiation was most prominent in the PC-3 lineage. It seemed that each prostate cancer cell line had limited differentiation potentials for the bone marrow mesenchymal stem cell lineage.

Though prostate cancer cells are known to have osteomimetic properties (12, 13), this study is the first to recognize their adipogenic potential. Subsequently, PC-3 lineaged cells and DU145 cells were used to characterize the phenomenon. These cells expressed a high level of PPAR\(\gamma\) (Figure 2B), the master regulator of adipogenic differentiation (25, 26). Together with the expression of downstream adipogenic markers FABP4/aP2 and PLIN1, these assays confirmed the adipogenic stem cell property. Adipogenic induction resulted in morphologic changes similar to brown fat cells (Figures 1B, 2A, and 4). In concordance to the induced expression of brown adipocyte-specific UCP1 (Figure 2B), these results strongly suggested that prostate cancer cells had differentiated into brown fat-like cells. Moreover, this study characterized the abnormally enhanced UCP1 expression as a unique marker for prostate cancer progression and bone metastasis.

Unlike white fat cells, brown adipocytes consume energy rather than serving as energy storage (25), while UCP1 protein uncouples oxidative phosphorylation from ATP synthesis to cause mitochondrial proton leak by dissipating energy as heat (34). Intriguingly, increased expression
of UCP1 has been postulated to be involved in cancer cachexia (35, 36). Further investigation into UCP1 may define the cause of cachexia during prostate cancer progression and metastasis.

This study revealed that the adipocyte-like cancer cells could reverse the differentiation program by retrodifferentiation, while the retrodifferentiated cells could be induced again to differentiate into adipocyte-like cells (Figure 4). On the other hand, we determined that adipocyte differentiation in the mMSC appeared to be an irreversible process (data not shown). It seemed that prostate cancer stem cells harbored extraordinary mechanisms empowering retrodifferentiation, which is an ancient mechanism invoking apoptosis escape and cell cycle re-entry (37). Considered as an approach to cellular rejuvenation and regeneration, retrodifferentiation has been explored as a treatment for degenerative and dystrophic diseases, but the underlying regulatory mechanism remains obscure. Retrodifferentiation is potentially a cause of prostate cancer recurrence, and the current study has established a model to illustrate its mechanism of molecular regulation.

Human prostate cancer cell lines responded differently to differentiation induction. Under adipogenic conditions, for instance, cells of the LNCaP lineage and DU145 cells responded slowly with limited lipid droplet production but sensitively with growth arrest. Cells of the PC-3 lineage showed opulent lipid droplets and acute apoptotic death. The mechanism underlying the differential sensibility to induced differentiation remains to be elucidated by comparative studies. Importantly, the adipogenic induction inhibited the growth of cancer cells (Figure 3A) and most adipocyte-like cancer cells in the PC-3 lineage died through apoptosis (Figures 3B and 3C), implying that induced differentiation could be an effective therapeutic strategy. Induced
Differentiation has been used to treat stem cell malignancies by pushing immature cells to differentiate into a more mature state (38, 39), since mature cells have limited life spans and would perish eventually through programmed mechanisms (29). Acute promyelocytic leukemia, for example, can be effectively treated with all-trans retinoic acid, a specific inducer for immature blood cells to differentiate and die (40). Further investigation is warranted to evaluate the adipogenic differentiation as a therapeutic modality for prostate cancer. In our study, the inducing agents for adipogenic differentiation were the glucocorticoid dexamethasone and the non-steroid anti-inflammatory drug indomethacin. Dexamethasone and indomethacin have been used as monotherapies for prostate cancer (41, 42), while their efficacy in combinatory use remains to be evaluated. We have observed synergistic effects of the combinatory use on PC-3 cell adipogenic differentiation. It would be interesting to determine whether an adipogenic differentiation therapy, in combination with novel strategies to block retrodifferentiation, can effectively inhibit prostate cancer recurrence and prevent metastasis.

It is paradoxical to notice that although in vitro UCP1 induction in prostate cancer cells, especially in the PC-3 lineage, was accompanied by growth inhibition and marked apoptosis (Figure 3); progressively enhanced UCP1 expression was correlated with malignant status in clinical prostate cancer specimens (Figure 5). Whether the UCP1 level is accompanied by growth inhibition and apoptosis in vivo remains to be determined. It becomes critical to simultaneously evaluate the adipogenic marker expression versus growth inhibition and apoptosis in individual clinical prostate cancer specimens. On the other hand, being a unique protein in mature brown fat cell mitochondria mediating energy dissipation, UCP1 may serve as
a brown adipocyte lineage marker, but may not be integral to growth arrest and programmed death, which could be controlled by mechanisms independent of UCP1 protein function.

The study of prostate cancer stem cells is at an early stage, and how mesenchymal stem cell properties come into existence in prostate cancer cells is presently unclear. RWPE-1 and PrEC cells were used in this study to represent normal prostate epithelial cells, and these cells could not be induced to undergo either osteoblastic or adipogenic differentiation (Figure 1), implying that the mesenchymal stem cell properties in the cancer cells were acquired abnormalities. This study revealed that a single prostate cancer cell line could have the stem cell properties of heterogeneous cell lineages. The extent of heterogeneity remains to be identified, but tumor cells might have opportunities to acquire the additional stem cell properties of multiple cell lineages during the chronic process of prostate cancer progression. Prostate cancer cells of the LNCaP lineage, for instance, could be induced to express neuroendocrine phenotypes, while clinical prostate cancer has been known for neuroendocrine differentiation (43). Although adipogenic induction alone may promote differentiation-induced programmed death of the cancer cells that harbor adipogenic stem cell properties, further investigations have to be conducted to search and define other stem cell properties that could be exploited for prostate cancer differentiation therapy.

**Conclusion**

This study revealed that prostate cancer cells could be induced to osteoblastic and adipogenic differentiation, while cancer cell adipogenic differentiation was along the brown-adipocyte
lineage. Lineage-specific markers were found in the majority of clinical prostate cancer specimens, suggesting that clinical prostate tumors harbor similar stem cell properties. Importantly, adipogenic differentiation led cancer cells to growth arrest and apoptotic death, arguing that this stem cell property can be exploited as a means of differentiation therapy. This report is thus the first to define the lineage specificity of human prostate cancer stem cells. Established prostate cancer cell lines with defined stem cell properties provide simple and reproducible models which are invaluable for studying the role of cancer stem cells in cancer progression and bone metastasis.

Acknowledgments

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Disclosure of Potential Conflicts of Interest

None disclosed.
References


Table 1. Pathologic characterization of the prostate cancer bone metastasis specimens.

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a. All cases were diagnosed with castration resistant prostate cancer.
b. Gleason of the primary cancer at diagnosis.
c. Years of post diagnosis survival.
d. Each score represent the staining results of more than 6 cores.
e. Final measurement of total PSA in circulation (ng/ml).
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Figure Legends

Figure 1. Prostate cancer cells harbor the properties of bone marrow mesenchymal stem cells. A, prostate cancer cells were treated in osteoblastic differentiation medium (Osteoblastic induction). B, prostate cancer cells were treated in adipogenic differentiation medium (Adipogenic induction). After a 21-day induction, cells cultured in control medium (Control) and the respective inducing media (Differentiated) were subjected to specific staining. hMSC was used as a positive control. The results are representative of three separate experiments. All microphotographs are at 100× magnification.

Figure 2. Characteristics of adipogenic differentiation. PC-3 human prostate cancer cells were treated with adipogenic differentiation medium. A, the treated PC-3 cells were assessed for adipogenic differentiation weekly by Oil Red O staining. All microphotographs are at 100× magnification. B, RT-PCR was used to assess expression of adipocyte-specific marker genes in the treated PC-3 cells. Total RNA samples from treated cells at different time points (days) were used as templates. λHindIII/ΦX174HaeIII was used as a DNA marker (M). In these studies, the results are representative of three separate experiments. Similar results were obtained when PC-3M cells were studied.

Figure 3. Effects of adipogenic induction on growth and survival of prostate cancer cells. A, Cells were cultured in control medium (Control) or treated with adipogenic differentiation medium (Induced). After 7 days of treatment, the cells were subjected to cell proliferation assay. Each data point represents the mean of a triplicate assay. B, PC-3 cells were cultured in control
medium (Control) or adipogenic differentiation medium (Induced). After 14 days of treatment, the cells were subjected to FACS analysis. The elevated subdiploid peak in the treatment group indicates death of the adipocyte-like cancer cells. Fraction of the subdiploid peak was determined from a triplicate assay. C, PC-3 cells were treated with adipocyte differentiation medium. The treated cells were sampled at different time points (days) for DNA fragmentation assay. In these studies, the results are representative of two separate experiments. An asterisk indicates statistical significance ($P < 0.05$).

**Figure 4. Adipogenic differentiation and retrodifferentiation in PC-3 cells.** This schematic presentation summarizes the results of repeated differentiation and retrodifferentiation. Annotations depicting the process of differentiation are shown in a dark color. Annotations used in retrodifferentiation are in grey. All the microphotographs are at 200× magnification. PC-3 cells could undergo consecutive adipogenic differentiation and retrodifferentiation for at least 3 cycles. Similar results were obtained from PC-3M and DU145.

**Figure 5. Enhanced adipogenic marker UCP1 expression in prostate cancer specimens.** A specific antibody was used to detect UCP1 expression in human prostate cancer specimens. Micrographic photos of representative results are shown. In 2 of the benign prostate tissues, UCP1 could be found preferentially in cells in the basal layer (A). In cancer-inflicted regions, higher staining was seen in glands with PIN lesion (B), and in both well-differentiated and poorly differentiated primary cancer cells (C). Still stronger UCP1 staining was found in prostate cancer bone metastasis (D). In many metastatic tumors, the stain appeared grainy, probably reflecting the stain of mitochondria (E), while in many others stained cells were filled
with organelles suggestive of lipid droplets (F). All the microphotographs are at 200 × magnification. The abnormally enhanced UCP1 expression level is plotted against each corresponding prostate cancer type (G). Asterisk indicates statistical significance compared to the level in normal prostate ($P < 0.05$).
Table 1. Pathologic characterization of the prostate cancer bone metastasis specimens.

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<td>++++</td>
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</table>

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Clinical Cancer Research

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