Prostate Cancer Cells Induce Osteoblast Differentiation through a Cbfal-dependent Pathway


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ABSTRACT

Metastases from prostatic adenocarcinoma (prostate cancer) are characterized by their predilection for bone and typical osteoblastic features. An in vitro model of bone metastases from prostate cancer was developed using a bicompartiment coculture system of mouse osteoblasts and human prostate cancer cells. In this model, the bone-derived prostate cancer cell lines MDA PCs 2a and MDA PCs 2b induced a specific and reproducible increase in osteoblast proliferation. Moreover, these cells were able to induce osteoblast differentiation, as assessed by increased alkaline phosphatase activity, Osteocalcin expression, and calcified matrix formation. This osteoblastic reaction was confirmed in vivo by intrafemoral injection of MDA PCs 2b cells into severe combined immunodeficiency disease mice. In contrast, the highly undifferentiated, bone-derived human prostate cancer cell line PC3 did not produce an osteoblastic reaction in vitro and induced osteolytic lesions in vivo. The osteoblast differentiation induced by MDA PCs 2b cells was associated with up-regulation of the osteoblast-specific transcription factor Cbfal. Moreover, treatment of osteoblasts with conditioned medium obtained from MDA PCs 2b cells resulted in up-regulation of Cbfal and Osteocalcin expression. In support of the differentiation studies, a microarray analysis showed that primary mouse osteoblasts grown in the presence of MDA PCs 2b cells showed a shift in the pattern of gene expression with an increase in mRNA encoding Procollagen type I and Osteopontin and a decrease in mRNA-encoding proteins associated with myoblast differentiation, namely myoglobin and myosin light-chain 2. Taken together, these findings suggest that the bone-derived prostate cancer cells MDA PCs 2a and MDA PCs 2b promote differentiation of osteoblast precursors to an osteoblastic phenotype through a Cbfal-dependent pathway. These results also established that soluble factors produced by prostate cancer cells can induce expression of osteoblast-specific genes. This in vitro model provides a valuable system to isolate molecules secreted by prostate cancer cells that favor osteoblast differentiation. Moreover, it allows us to screen for therapeutic agents blocking the osteoblast response to prostate cancer.

INTRODUCTION

Prostate cancer is currently recognized as the most frequent form of cancer in males and the second leading cause of cancer death in men in the United States (1). Although localized prostate cancer may be cured, 70% of patients with metastases will die of cancer rather than an unrelated cause (2, 3). Characteristically, the metastatic dissemination pattern of prostate cancer is: (a) the spread has a predilection for bone in ~80% of cases; and (b) bone metastases are typically osteoblastic (4). Usually bone metastases are initially sensitive to testosterone deprivation, but given sufficient time, androgen-independence eventually occurs in all cases. This latter situation is associated with bone complications and carries a poor prognosis, with median survival of <1 year. This strongly suggests that the interaction of prostatic cancer cells with cells of the osteoblast lineage contributes to the lethal progression of prostate cancer, although the molecular nature of this interaction is still poorly understood. In particular, it is not known whether this interaction requires cell-to-cell contact. The classical “seed-and-soil” hypothesis proposes that neoplastic cells prefer to colonize an organ that may serve as fertile soil (5). One interpretation of this hypothesis is that prostate cancer cells may be specifically attracted by factors released from bone and, thus, migrate preferentially to it (6). Another plausible explanation involves the osteomimetic properties of prostatic metastases to support their predilection to bone (7).

One major hindrance in the study of the biology of metastatic prostate cancer has been the limited number of laboratory models of prostate cancer, compared with the number of models available for other neoplasms (8, 9). Indeed, no reliable in vitro model of osteoblastic bone metastasis from prostate cancer is currently available. Thus, establishing a reliable model of bone metastases would be important. This would allow us to have a better understanding of its biology, to study at the molecular level the interaction of prostate cancer cells with osteoblasts, to develop more efficient therapies (especially bone-targeted therapies), and to further investigate the mechanisms of resistance to drugs in bone.

We previously established two prostate cancer cell lines: MDA PCs 2a and MDA PCs 2b (10, 11). These cell lines are the first ones derived from a bone metastasis of prostate cancer to possess typical features of prostate cancer, because they express PSA,2 the androgen receptor, and their proliferation is regulated by androgens. In this study, we cocultured MDA PCs 2a and MDA PCs 2b with mouse osteoblasts. We established and optimized this in vitro model of bone metastases from prostate cancer and showed evidence that these cells induced a specific increase in osteoblast growth and differentiation. We also demonstrated that these biological events are associated with an increase in expression of Cbfal, Procollagen type I, Osteocalcin, and Osteopontin, whereas the expression of genes associated with myoblast differentiation was repressed.

MATERIALS AND METHODS

Cell Culture. MDA PCs 2a and MDA PCs 2b cell lines (11) were routinely propagated in BRFF-HP1 medium (Biological Research Faculty and Facility, Inc., Jamsville, MD) with 20% FBS (Life Technologies, Inc., Gaithersburg, MD). LNCaP, PC3, HeLa, T24, ROS-17/2.8, and CV-1 cell lines were obtained from the American Type Culture Collection (Rockville, MD). LNCaP was maintained in RPMI 1640, ROS-17/2.8 was maintained in DMEM/F12K, and the other cell lines were maintained in DMEM. All were supplemented with 10% FBS. LNCaP and PC3 were routinely propagated in BRFF-HPC1 medium (Biological Research Faculty and Facility, Inc., Jamsville, MD).}

Received 3/1/01; accepted 5/31/01.

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1 Supported by CaP CURE, DAMD17-99-1-9028, NIH Grant CA75499-04, DK 50583, and the Ligue contre le cancer.
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4 The abbreviations used are: PSA, prostate-specific antigen; FBS, fetal bovine serum; PMO, primary mouse osteoblast; CM, conditioned medium; α-MEM, α-modified Eagle medium.
mouse preosteoblast cell line 2T3 (12) was a generous gift from Stephen Harris, University of Texas Health Science Center, San Antonio, TX. These cells were grown in α-MEM (Life Technologies, Inc.) plus 10% FBS.

Primary Cultures of Mouse Calvaria Osteoblasts. To obtain primary culture of osteoblasts, we used newborn CD1 mice that were killed 4 days after birth. Cultures of PMOs were obtained from the calvaria by use of a procedure published previously (13), with slight modifications. In brief, after dissection, calvaria were digested for 15 min in a shaking incubator at 37°C in 15 ml of α-MEM containing 0.1 mg/ml collagenase P (Boehringer Mannheim, Corp., Indianapolis, IN), 2.5% trypsin/EDTA (Life Technologies, Inc.), streptomycin, and penicillin. The mixtures were also gently shaken by hand for 20 s every 5 min during the procedure. The digestion medium and any released cells were then discarded. This entire procedure was repeated for another 15 min, and the medium was discarded. Finally, it was found that if the cells were digested for 15 min in differentiation medium for 8 days, and alkaline phosphatase activity and osteocalcin introduction were assessed. Each experiment was performed in triplicates.

Coculture of Immortalized Murine Osteoblasts (2T3 Cells) with Prostate Cancer Cells. We tested the reproducibility of our findings by using a well-characterized clonal osteoblast cell line, 2T3, derived from mouse calvaria (12). PMOs were replaced in the model by the 2T3 cells. In these experiments, 1000 cells/cm² 2T3 cells were cocultured with MDA PCa 2a, MDA PCa 2b, or LNCaP cells under the conditions already described. After 4 days of coculturing, the number of 2T3 cells was assessed. As described for PMOs, the 2T3 cells were allowed to differentiate again in differentiation medium for 8 days, and alkaline phosphatase activity and osteocalcin introduction were assessed. Each experiment was performed in triplicates.

Alkaline Phosphatase Activity and Osteocalcin Secretion Level. Alkaline phosphatase activity was determined in cell extracts with the Sigma Chemical Co. Diagnostics alkaline phosphatase reagent (St. Louis, MO). The level of osteocalcin in the culture medium was determined using a mouse osteocalcin immunoradiometric assay kit (Immunotopics, Inc., San Clemente, CA).

Mineralized Bone Matrix Formation Assay. Bone cell differentiation was monitored by using an assay for mineralized matrix formation (15), with slight modifications. In brief, von Kossa’s staining of mineralized bone matrix was performed as follows: cell cultures were washed twice with PBS, fixed in phosphate-buffered formalin for 10 min, and washed with water. Remaining water was removed, a 5% silver nitrate solution was added, and plates were incubated in bright light for 20 min. The reaction was stopped by rinsing the plates in water. Finally, 5% sodium thiosulfate was added, and the plates were rinsed twice with water.

CM Preparation and PMO Treatment. To assess the effect of soluble factors produced by MDA PCa 2b cells on gene expression of PMOs, we obtained CM from MDA PCa 2b cells. MDA PCa 2b cells were grown in -MEM plus 10% FBS for 48 h. The cells were then subsequently trypsinized and replated in culture dishes to perform experiments.

Cbfal Transcriptional Activity. To examine transcriptional activity mediated by Cbfal, we transfected PMOs with a reporter gene containing the multimerized Cbfal response element (OSE2; 5′-GATCCGGTCAATCAG-3′; Ref. 14) inserted upstream from the minimal promoter of the firefly luciferase expression vector pTLuc (Promega, Corp., Madison, WI). The cells were plated at a density of 5,000 cells/cm² in 35-mm dishes of the firefly luciferase expression vector pTAL-Luc (Promega, Corp., Madison, WI). The cells were treated with a 3:1 mixture of Lipofectamine reagent (Life Technologies, Inc.) and DNA for 5 h, at which time FBS was added to a final concentration of 10%. The medium containing the transfection reagent was removed after transfection with CCLR lysis buffer (Promega, Corp.) and assayed with a luminometer (TD 20/20; Turner Designs Inc., Sunnyvale, CA) and normalized for protein concentration in each cell lysate. Each transfection experiment was performed in triplicate, with pTLuc as a control, to determine the up-regulation of the minimal promoter activity by the multimerized OSE2 enhancer (5xOSE2-pTLAL construct). To examine the cell specificity of the OSE2-mediated transcription, we performed similar transfection experiments with the rat osteosarcoma cell line ROS-17/2.8 (positive control) and the monkey kidney cell line CV-1.

Coculture of PMOs with Human Cancer Cells. An in vitro bicompartiment culture system was developed as a model of bone metastases from prostate cancer. PMOs were seeded in tissue culture plates, whereas MDA PCa 2a or MDA PCa 2b were seeded in cell-culture inserts (0.4-μm pore; Falcon/Becton Dickinson Labware, Franklin Lakes, NJ). Coculturing was performed with α-MEM plus 5% BTFF-HPC1 as a growth medium for all cell types used in the inserts. A similar method was used to coculture LNCaP, PC3, HeLa, and T24 with PMOs. LNCaP was derived from a lymph node metastasis, not from a prostate cancer. PMOs were seeded in tissue culture plates, whereas MDA PCa 2a, MDA PCa 2b, LNCaP, and PC3 cells. After 4 days of coculturing, the inserts containing the prostate cancer cells were removed, and the PMOs were maintained in culture until they reached confluence (day 0). The PMOs were subsequently placed in differentiation medium (α-MEM plus 10% FBS, 100 μg/ml ascorbic acid, and 10 nm sodium β-glycerophosphate) in the presence of BMP-2 (20 ng/ml). PMOs growing in these conditions were tested for alkaline phosphatase activity every 2 days, from days 4 to 12. Calcified matrix formation was assessed by von Kossa staining at day 16 of culture in differentiation medium. Each experiment was performed in duplicates.

Bone Cell Differentiation. PMOs growing in these conditions were tested for alkaline phosphatase activity every 2 days, from days 4 to 12. Calcified matrix formation was assessed by von Kossa staining at day 16 of culture in differentiation medium. Each experiment was performed in duplicates.
The tumors were prepared by the Department of Veterinary Medicine of M. D. Anderson Cancer Center. The subject bones were dissected free of muscle, fixed in 10% buffered formalin, decalcified in 5% formic acid, and then embedded in paraffin. Longitudinal 3-μm-thick sections were obtained from each sample and stained with H&E.

**Statistical Analysis.** Numeric data were expressed as means ± SE. Statistical differences between means for the different groups were evaluated with Sigma Chemical Co. Plot 4 one-way ANOVA and Tukey’s mean separation test, with the level of significance set at \( P < 0.05 \).

**RESULTS**

**PMOs Express Active Cbfa1 Transcription Factor.** We assessed if Cbfa1 expressed in PMOs was transcriptionally active. Cbfa1 is a gene encoding a transcription factor whose function is critical for osteoblast differentiation, and it is the earliest known molecular marker of osteogenesis. Cbfa1 is expressed in cells destined to become osteoblasts but not in any other cells at any significant level (18). We found previously that Cbfa1 RNA is expressed in PMOs (19). Fig. 1B shows that the Cbfa1 response element (OSE2) increased the minimal promoter activity of the reporter pTAL by 15- to 20-fold in PMOs and 5- to 6-fold in the osteoblast-like ROS-17/2.8 cells. In contrast, there was no detectable activity in the CV-1 kidney cell line. These results confirm the cell specificity of OSE2-mediated transcription and suggest that the cells isolated from calvaria contain a high

![Diagram of luciferase activity](attachment:image)

**Intrabone Injections.** An intrabone injection model of bone metastasis was developed to confirm in vivo the osteoblastic phenotype induced by the MDA PCa 2b cells in vitro. Male severe combined immunodeficiency disease mice obtained from Charles River Breeding Laboratories (Wilmington, MA) were housed in a facility with constant humidity and temperature and a 12-h light-dark cycle. They had ad libitum access to standard mouse feed and water and were monitored daily. Animals were anesthetized with i.m. injections of ketamine 100 mg/kg plus acepromazine 2.5 mg/kg. An average number of 1.2 \( \times 10^6 \) MDA PCa 2b or PC3 cells was diluted in 5 μl of growth medium, and then 13 mice were injected into the right femur (or tibia) of each mouse. The same volume of growth medium was injected into the left femur of each mouse as a control. Mice were then monitored twice weekly for tumor bulk and tested biweekly for PSA serum levels. Radiographs of the bones that had received the injections were obtained every month and before the mice were killed. Animals were killed after 3 months (less in case of a bulky tumor), and a pathological examination of the subject bones was performed.

**PSA Blood Levels.** Blood from the mice was obtained at regular intervals from a small incision in the main tail vein. Serum was separated from the blood, and PSA was measured using a microparticle enzyme immunoassay (IMx PSA assay, Abbott Laboratories, Abbott Park, IL).

**Tissue Samples.** Formalin-fixed, paraffin-embedded tissue samples from the tumors were prepared by the Department of Veterinary Medicine of M. D.

amount of transcriptionally active Cbfa1, and therefore, they are likely to include osteoblasts or osteoblast precursors.

Specificity of PMO Proliferation Induced by Coculturing with MDA PCa 2a and MDA PCa 2b Cells. Evidence of a significant increase in PMO proliferation was found when these cells were cocultured with MDA PCa 2a and MDA PCa 2b cells ($P < 0.001$ for both, Fig. 2). This increase was confirmed in four consecutive experiments and was always consistent. When PMOs were cocultured with LNCaP cells, a proliferative response was also found in the PMOs, although the magnitude of this response varied between experiments. In contrast, when PMOs were cocultured with PC3 cells, the number of PMOs decreased significantly ($P < 0.001$). Moreover, the number of PMOs also decreased significantly after coculturing with HeLa cells ($P < 0.001$), whereas no significant modification was observed with T24 cells (Fig. 2). These results suggest that the proliferative response observed with the MDA PCa 2a and MDA PCa 2b bone-derived prostate cancer cells is not universal and may be specific.

Coculturing with Prostate Cancer Cells Promotes Differentiation of PMOs. Cells isolated from calvaria comprise osteoblasts at multiple stages of differentiation. In prolonged culture, these cells undergo a defined series of events from proliferation to maturation, express Osteopontin, Alkaline phosphatase, Bone sialoprotein, and Osteocalcin genes, and ultimately form mineral (20–24). Of those four genes, only the Osteocalcin genes are expressed solely in osteoblasts and no other extracellular matrix-producing cell (18). To assess if the prostate cancer cells could induce differentiation of osteoblast cells, we studied Osteocalcin expression in the PMOs grown alone and 4 days of coculturing with MDA PCa 2b. As Fig. 3 shows, Osteocalcin expression was increased in PMOs that had been grown in the presence of MDA PCa 2b cells compared with PMOs grown alone. Cbfa1 is a central regulator of osteoblast differentiation and function and, as such, activates most of the genes expressed by osteoblasts (18). Fig. 3 also shows that Cbfa1 expression is up-regulated in PMOs cocultured with MDA PCa 2b cells. It is noteworthy that up-regulation of Cbfa1 transcripts was not always observed when Osteocalcin was induced in cocultured PMOs. After PMOs and prostate cancer cells had been separated, a 2-fold increase in alkaline phosphatase activity was seen in PMOs that had been grown with MDA PCa 2a or MDA PCa 2b cells. In contrast, decreased alkaline phosphatase activity was detected when PMOs had been cocultured with PC3 cells, compared with PMOs grown alone (Fig. 4). Von Kossa staining (Fig. 5) shows an increase in calcified matrix forma-
tion in PMOs that had been cocultured with MDA PCa 2a or with MDA PCa 2b, compared with PMOs grown alone. PMOs cocultured with LNCaP cells did not induce calcified matrix formation, and PMOs cocultured with PC3 cells exhibited a decrease in calcified matrix formation, compared with controls. This suggests that PC3 cells secrete molecules that inhibit the osteoblast differentiation program. These results agree with those of others (25, 26) and further validate this system.

Soluble Factors Produced by MDA PCa 2b Cells Induced Cbfal and Osteocalcin Expression in PMOs. MDA PCa 2b cells were able to induce expression of markers of osteoblast differentiation by PMOs in the coculture system. Because in this coculture system, prostate cancer cells and PMOs are not in physical contact, it is likely that soluble factor (or factors) secreted by these prostate cancer cells mediates this effect. We therefore assessed the effect of CM produced by MDA PCa 2b cells on the expression of Cbfal and Osteocalcin by PMOs. Fig. 6A shows a substantial increase in Osteocalcin expression after 6 h of PMOs grown in crude CM. When PMOs were grown for 12 h in α-MEM plus 3 × CM7a, a substantial increase in Cbfal and Osteocalcin expression was observed (Fig. 6B). These results indicate that soluble factors produced by MDA PCa 2b cells induce expression of Cbfal and in turn Osteocalcin, a target of Cbfal.

Coculturing with Prostate Cancer Cell Lines Induces Proliferation and Differentiation of Immortalized Mouse Osteoblasts (2T3 Cells). Results are summarized in Fig. 7. Increased 2T3 proliferation, alkaline phosphatase activity, and osteocalcin production were detected when these cells were cocultured with MDA PCa 2a and MDA PCa 2b cells, compared with controls. A significant increase in 2T3 growth, but no increased alkaline phosphatase activity or osteocalcin production was detected when these cells were cocultured with MDA PCa 2a or osteoblasts. None of the control mice tested had blood PSA levels >0.2 ng/ml Bone lesions were monitored by X-ray at days 60, 80, and 100. Three mice died before X-ray was performed. Of the remaining 10 mice, 5, 6, and 8 developed bone (mostly osteoblastic) lesions at days 60, 80, and 100, respectively, after the injection. The contralateral legs never showed evidence of osteoblastic lesions. The mice also exhibited a significant increase in PSA levels exceeding 4.5 ng/ml

Table 1 Gene expression of extracellular matrix genes

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Balanced differential expression</th>
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<tbody>
<tr>
<td>Mus musculus mRNA for collagen α1(V)</td>
<td>6.0</td>
</tr>
<tr>
<td>Procollagen, type XI, α 1</td>
<td>9.5</td>
</tr>
<tr>
<td>Procollagen, type III, α 1</td>
<td>9.5</td>
</tr>
<tr>
<td>Procollagen, type VI, α 1</td>
<td>11.2</td>
</tr>
<tr>
<td>Secreted phosphoprotein 1 (Osteopontin)</td>
<td>12.3</td>
</tr>
<tr>
<td>Procollagen, type V, α 2</td>
<td>18.9</td>
</tr>
<tr>
<td>Procollagen, type 1, α 1</td>
<td>22.5</td>
</tr>
</tbody>
</table>

* Internet address: www.mdanderson.org/PMOGeneExpression.
itted features of bone resorption, primarily at the injection site (Fig. 9). One mouse had a rapidly growing soft tissue tumor and died soon after the first X-ray, and only 1 had no evidence of tumor. Nineteen control mice had $1 \times 10^6$ PC3 prostate cancer cells injected under the same conditions. X-ray was performed in every mouse 30–60 days after the procedure. None of the 14 mice that survived the procedure showed evidence of new bone formation when the bones had been injected with MDA PCa 2b. The left side of Fig. 10 shows a tumor produced by PC3 cells growing in the marrow cavity of a severe combined immunodeficiency disease mouse. The tumor has destroyed cortical bone and is growing into the muscle outside the bone. In the right side of Fig. 10, higher magnification reveals the randomly deposited basket-weave pattern that is characteristic of new bone formation. Surrounding the new bone is the lamellar (mature) bone. In contrast with what occurred with MDA PCa 2b cells, osteoclast recruitment and bone destruction were obvious in bones that had been injected with PC3 cells (Fig. 10C). The left side of Fig. 10C shows a tumor produced by PC3 cells growing in the narrow cavity of a severe combined immunodeficiency disease mouse. The tumor has destroyed cortical bone and is growing into the muscle outside the bone. In the right side of the same panel, higher magnification allows identification of several osteoclasts, a characteristic finding in bone injected with PC3 cells.

**DISCUSSION**

Currently, no model is available to study the cellular and molecular events associated with bone metastases of prostate cancer. This study shows that MDA PCa 2a and MDA PCa 2b cells induce a specific and reproducible increase in osteoblast differentiation and proliferation when the cells share the medium during coculturing. Osteoblast differentiation in this system was associated with up-regulation of the osteoblast-specific transcription factor Cbfa1. Moreover, up-regulation of Cbfa1 and Osteocalcin expression was also induced in PMOs cocultured with MDA PCa 2b cells, which suggests that Cbfa1 mediates osteoblast differentiation in this system. Increased expression of Cbfa1 transcripts was not always observed in cocultured PMOs when Osteocalcin was up-regulated. Recently, it has been proposed that Cbfa1 transcriptional activity be regulated directly by transcription factors, that Cbfa1 may need to be activated by posttranscriptional modifications, and that Cbfa1 function could be modulated by cofactors (28); therefore, it is likely that Cbfa1 activation occurs in the absence of up-regulation of its transcripts. Gene expression analysis of PMOs grown in the presence of MDA PCa 2b cells showed that soluble factors produced by these prostate cancer cells induced a shift in the expression of transcripts, resulting in increased expression of extra-cellular matrix genes and decreased expression of genes related to myoblast differentiation. Osteoblasts are derived from common mesenchymal progenitors that can also differentiate into chondroblasts, myoblasts, and adipocytes (29–31). Moreover, some clones derived from rat calvaria osteoblasts have been reported to exhibit both osteoblast-like phenotypes and notable formation of myotubes (30, 31).

This indicates that osteoblasts derived from neonatal rodent calvaria are in different stages of differentiation and developmental commitment, which probably accounts for the expression of Myosin light chain 2 and Myoglobin in PMOs. The possibility of contamination by myoblasts from surrounding muscle in PMO cultures is unlikely because the procedure was designed and validated to remove nonbone cells (13). The decreased levels of transcripts of genes related to myoblast differentiation in PMOs after coculturing with MDA PCa 2b cells are more likely the result of the enrichment of the PMOs either by increased proliferation of cells of the osteoblast lineage or by selective inhibition of myoblast differentiation. Myogenic differentiation has been reported to be inhibited by factors such as BMP-2, transforming growth factor-β, and basic-fibroblast growth factors (32–34). Moreover, BMP-2 has been shown to convert differentiation of myoblasts into the osteoblast lineage (35). Human prostate cancer cells have been shown to produce several growth regula-

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**Table 2. Expression of genes related to myoblast differentiation**

<table>
<thead>
<tr>
<th>Balanced differential expression</th>
<th>Gene name</th>
<th>Reference</th>
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<tr>
<td>$-24.8$</td>
<td>Mas musculus myosin light chain 2</td>
<td>Xu et al., 2000 (47)</td>
</tr>
<tr>
<td>$-10.8$</td>
<td>Muscle glycogen phosphorylase</td>
<td>Froman et al., 1998 (48)</td>
</tr>
<tr>
<td>$-11.9$</td>
<td>Mas musculus mRNA for stretch-regulated skeletal muscle protein (Uoxmd gene)</td>
<td>Xu et al., 2000 (47)</td>
</tr>
<tr>
<td>$-10.7$</td>
<td>Myoglobin</td>
<td>Garay et al., 2000 (49)</td>
</tr>
<tr>
<td>$-6.2$</td>
<td>Enolase 3, β muscle</td>
<td>Keller et al., 1992 (50)</td>
</tr>
<tr>
<td>$-6.9$</td>
<td>Ryanodine receptor 1, skeletal muscle</td>
<td>Ogawa et al., 1999 (51)</td>
</tr>
</tbody>
</table>

**Fig. 8. Northern blot analysis of expressed genes in PMOs grown alone (PMO control) or after 4 days of coculturing with MDA PCa 2a, MDA PCa 2b, LNCaP, or PC3 cell lines.**

A. **Procollagen type I expression**; B. **Osteopontin expression**; C. **Myosin light chain 2 expression**; D. **Myoglobin expression**. 36B4 was used as a loading control.
tory factors, including transforming growth factor-β and basic-fibroblast growth factors, as well as bone morphogenetic proteins (35), again suggesting that BMP-2 may be a player in the osteoinductive effect of prostate cancer cells.

Our findings of an osteoinductive effect of MDA PCa 2a and MDA PCa 2b cells in vitro were reproducible in an in vivo model. Osteoclasts were scarce in tumors produced by MDA PCa 2b cells, whereas tumors produced by PC3 cells resulted in osteolytic lesions and substantial recruitment of osteoclasts. Until recently, our insight into bone-metastasis biology was based mostly on the study of prostate cancer cells. These studies were limited because: (a) the bone compartment was not studied; and (b) the cell lines used (mainly LNCaP, PC3, and DU-145) do not fully reflect the common biological features of bone metastasis from prostate cancer (8). Geldof and Rao (36) have reported that injection of R3327-MatLyLu rat tumor cells into the tail vein, with concomitant vena cava occlusion, results in skeletal metastases, although these lesions are osteolytic. Finally, Wu et al. (37) have reported that two of seven animals developed osteoblastic bone metastases after receiving intracardiac injection of C4–2 cells derived from LNCaP. However, because the growth of C4–2 cells is not regulated by androgen, we believe our system provides the in vitro model counterpart to these models and will be useful in identifying bone metastasis-related genes, osteoblast-stimulating factors, or both, which might be more relevant to the natural history of metastatic prostate cancer in humans.

Our work confirms that modulation of osteoblast proliferation by prostate cancer cells occurs without any physical contact. Gutman et al. (38) first hypothesized the production of osteoblast-stimulating factors by prostate cancer, and our results agree thus far. Indeed, PSA (39, 40), urokinase (41, 42), bone morphogenic proteins (43, 44), and endothelin-1 (45) have been identified as direct or indirect osteoblast-stimulating factors expressed by prostate cancer cells. Of note, the role of some of these factors (41, 46) has been established by use of the conditioned culture medium of PC3, a cell line that does not reflect the common biological features of prostate cancer, because it is minimally differentiated and does not produce PSA. Our work confirms that PC3, in contrast to MDA PCa 2b, does not produce osteoblastic lesions in vivo but actually produces osteolytic lesions.

In contrast to the consistent and reproducible induction of osteoblastic growth observed in our in vitro model, we often saw increased proliferation of MDA PCa 2a and MDA PCa 2b cells, but it did not always reach statistical significance. These results might indicate that other cells from the bone compartment are required to provide a growth advantage to prostate cancer cells. The conditions under which we performed these experiments were set to optimize the study of PMO growth and differentiation, but they might not have been optimal for studying the proliferative response of prostate cancer cells in the presence of PMOs. Finally, the short duration of the coculturing in our system may be an alternative explanation for the apparent lack of a reproducible increase in prostate cancer cell growth, in terms of their well-recognized slow growth.

In summary, our results describe the establishment, optimization, and molecular analysis of an in vitro model of bone metastases from prostate cancer. MDA PCa 2a and MDA PCa 2b cells induced the osteoblastic features of bone metastases observed in the clinic, and this effect was mediated by increased osteoblastic growth and differentiation. This model proved valuable for studying molecular mechanisms underlying the interactions of prostate cancer and bone. It may also represent an attractive system for identifying molecular targets, on either the malignant compartment or the osteoblast compartment that may prevent the growth of prostate cancer cells in bone.
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