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Prostate Cancer Cells Induce Osteoblast Differentiation through a *Cbfa1*-dependent Pathway[1]

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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 bicompartment coculture system of mouse osteoblasts and human prostate cancer cells. In this model, the bone-derived prostate cancer cell lines MDA PCa 2a and MDA PCa 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 PCa 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 PCa 2b cells was associated with up-regulation of the osteoblast-specific transcriptor factor *Cbfa1*. Moreover, treatment of osteoblasts with conditioned medium obtained from MDA PCa 2b cells resulted in up-regulation of *Cbfa1* and *Osteocalcin* expression. In support of the differentiation studies, a microarray analysis showed that primary mouse osteoblasts grown in the presence of MDA PCa 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 PCa 2a and MDA PCa 2b promote differentiation of osteoblast precursors to an osteoblastic phenotype through a *Cbfa1*-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 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-independent growth 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 osteoblast, 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 PCa 2a and MDA PCa 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,[4] the androgen receptor, and their proliferation is regulated by androgens. In this study, we cocultured MDA PCa 2a and MDA PCa 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 *Cbfa1*, *Procollagen type I*, *Osteocalcin*, and *Osteopontin*, whereas the expression of genes associated with myoblast differentiation was repressed.

MATERIALS AND METHODS

Cell Culture. MDA PCA 2a and MDA PCA 2b cell lines (11) were routinely propagated in BRFF-HPC1 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, the other cell lines were maintained in DMEM (Life Technologies, Inc.), and all were supplemented with 10% FBS. The immortalized

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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 again. Finally, it was repeated again for 25 min, and the cell suspensions were transferred to new tubes and washed with α-MEM plus 10% FBS. This last procedure was repeated three times; each time, the cell suspensions were collected and pooled. The cells were then plated in α-MEM plus 10% FBS for 48 h. The cells were then subsequently trypsinized and replated in culture dishes to perform experiments.

Cbfa1 Transcriptional Activity. To examine transcriptional activity mediated by Cbfa1, we transfected PMOs with a reporter gene containing the multimerized *Cbfa1* response element (OSE2; 5′-GATCCGCTGCAATCACCAACCACAGCA-3′; Ref. 14) inserted upstream from the minimal promoter of the firefly luciferase expression vector pTAL-Luc (Promega, Corp., Madison, WI). The cells were plated at a density of 5,000 cells/cm^2 in 35-mm dishes in α-MEM containing 10% FBS 24 h before transfection. On the day of transfection, the medium was changed to serum-free α-MEM, and 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 24 h and replaced with fresh medium containing α-MEM and 10% FBS. For measurements of luciferase activity, the transfected cells were lysed 72–96 h after transfection with CCLR lysis buffer (Promega, Corp.) and assayed with the luciferase assay reagent, according to the manufacturer's instructions (Promega, Corp.). Light units were measured with a luminometer (TD 20/20; Turner Designs Inc., Sunnyvale, CA) and normalized for protein concentrations in each cell lysate. Each transfection experiment was performed in triplicate, with pTAL-Luc as a control, to determine the up-regulation of the minimal promoter activity by the multimerized OSE2 enhancer (5xOSE2-pTAL 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* bicompartment 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% BRFF-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 is derived from a lymph node metastasis, not from a bone metastasis. PC3 is derived from a bone metastasis but does not produce PSA and is not regulated by androgens. HeLa and T24, two neoplastic cell lines not from prostate origin, were used as additional controls. We optimized this coculture model for the optimal number of cells to be seeded so that the cells would reach 80% confluence after 4 days of culturing. The optimal numbers of cells selected were as follows: (*a*) MDA PCa 2a or MDA PCa 2b, 20,000 cells/cm^2; (*b*) LNCaP, 10,000 cells/cm^2; (*c*) PC3, 1,000 cells/cm^2; (*d*) HeLa, 1,000 cells/cm^2; (*e*) T24, 750 cells/cm^2; and (*f*) PMO, 5,000 cells/cm^2. After 24 h of cell culturing, the inserts were placed into tissue-culture plates containing the PMOs so that the two different cell types shared the culture medium but were not in physical contact. Each experiment was assayed six times. The medium was changed every 2 days.

To assess if prostate cancer cells affected the ability of PMOs to differentiate after PMOs and prostate cancer cells had been separated, PMOs were cocultured in our system both without (control) and with 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.

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/cm^2 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 then grown in differentiation medium for 8 days, and alkaline phosphatase activity and osteocalcin production 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 100-mm tissue culture dishes until 60–70% confluent. We subsequently washed them with PBS and changed the medium to serum-free α-MEM. After 48 h, the CM was collected and filtered through a 0.2-μm sterilizing filter. This crude CM was used as PMO growth medium. After 6 h, PMOs were collected, the RNA was isolated, and a Northern blot analysis was performed.

The CM prepared as described was also concentrated by ammonium sulfate precipitation: 70 g of ammonium sulfate were dissolved in 100 ml of CM, the procedure was performed at 4°C, and the solution was stirred until dissolution was complete. The solution was then centrifuged at 10,000 g × 45 min, the supernatant was discarded, and the pellet (dissolved in a minimal volume) was dialyzed overnight at 4°C against 0.005 M 4-morpholinepropanesulfonic acid with 500-kDa cutoff membrane. The dialyzed product was recovered in a volume of 2.4 ml (~40-fold concentration of the original CM); we refer to this medium as CM_{70}.

PMOs were then grown in α-MEM plus CM_{70}, supplemented with 10% FBS. CM_{70} was added to the growth medium to produce a final concentration of three times the crude CM (3 × CM_{70}). After 12 h, PMOs were collected, RNA was isolated, and a Northern blot analysis was performed.

RNA Extraction and Northern Blot Analysis. Total RNA was extracted from PMO cell monolayers by using the RNAzol B Reagent (Biotecx Laboratories, Inc. Houston, TX). Then the RNA (20 μg) was denatured, and electrophoresis was conducted in sample buffer (50% formamide, 10% 4-morpholinepropanesulfonic acid buffer, 18% formaldehyde, 10% glycerol, 0.5% bromphenol blue, and 1 μg of ethidium bromide) on agarose-formaldehyde gel (80 V, 3.5 h). Finally, the RNA was transferred to nylon membranes (Bio-Rad Laboratories, Hercules CA) via passive transfer overnight and then cross-linked by UV transillumination. The membranes were prehybridized at 65°C for 3 h in a Rapid-Hyb buffer (Amersham Pharmacia Biotech, Piscataway, NJ) plus 1 mg/ml denatured salmon sperm DNA. Specific cDNA probes were purified and labeled with [α^{32}]P dCTP with a random primer labeling kit (Megaprime DNA Labeling System; Amersham Pharmacia Biotech). Hybridization with the probes *Myoglobin* (clone ID #738009), *Myosin light chain 2* (clone ID #466382; Incyte Genomics, Palo Alto, CA), *Cbfa1*, *Osteocalcin*, *Osteopontin*, and *Procollagen, type I α 1* (16) was carried out overnight at 65°C. The membranes were washed three times at the same temperature with decreasing concentrations of SSC (2×, 0.5×, and 0.1×) in 0.5% SDS. The blots were then exposed to Kodak X-OMAT film at −80°C and developed in

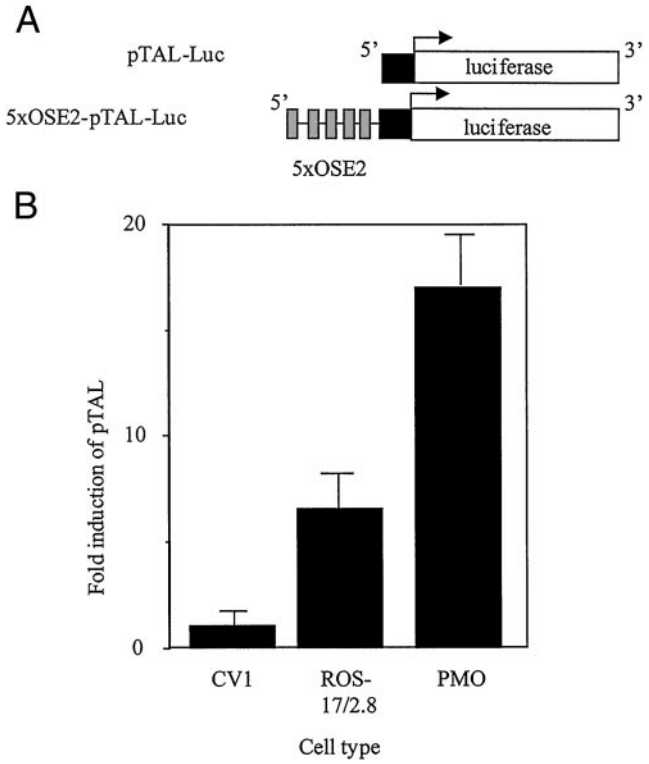

Fig. 1. Transcriptional activity of *Cbfa1* in PMOs. *A*, PMO, ROS 17/2.8, and CV-1 cells were transfected with the reporter 5xOSE2-pTAL-Luc or its control (pTAL-Luc) *B*, and reporter gene expression was measured 72 h later. Results are expressed as fold induction of pTAL-Luc activity by the OSE2 enhancer. Each transfection experiment was performed in triplicate. The transfection experiments with PMO or with ROS 17/2.8 cells were performed four times and were also performed twice with CV-1 cells.

an automatic film processor. Blots were also stripped and reprobed with a cDNA probe for 36B4 (17) or *Gapdh* as a control for even loading.

Gene Array Analysis. The differential expression of multiple genes in PMOs grown alone compared with that in PMOs grown in the presence of MDA PCa 2b cells was analyzed with the GEM 1 Gene Expression Microarray (Incyte Genomics). RNA was isolated from PMOs grown either alone (control) or cocultured with MDA PCa 2b. Poly(A)+ RNA preparation, cDNA probe synthesis, hybridization with Mouse GEM 1 cDNA microarray, and signal analysis were conducted by Incyte Genomics.[5]

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 1.2×10^6 of 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. 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. 1*B* 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

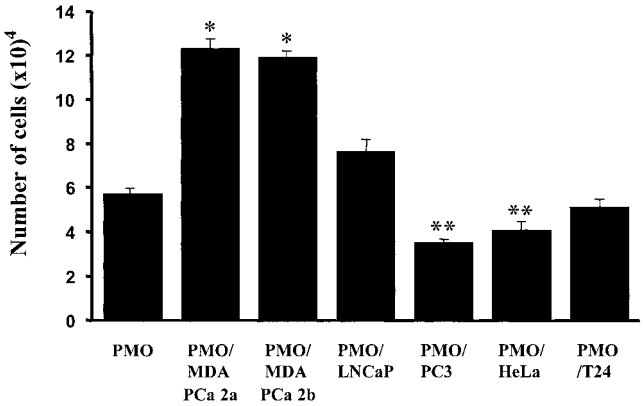

Fig. 2. Number of cells in PMO cultures grown for 4 days either alone (*PMO*) or in coculture with MDA PCa 2a, MDA PCa 2b, LNCaP, PC3, HeLa, and T24 cells. Each culture or coculture was assayed six times. Cells were counted in a hematocytometer. Results obtained from three different experiments are expressed as mean; *bars*, SE. *, cell number of cocultured PMOs significantly higher than controls ($P < 0.001$). **, cell number of cocultured PMOs significantly lower than controls ($P < 0.001$).

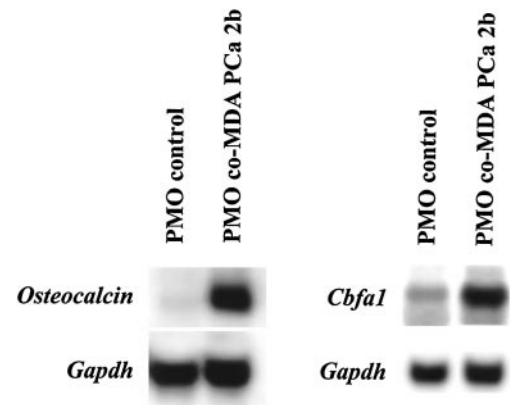

Fig. 3. Northern blot analysis of *Cbfa1* and *Osteocalcin* expression in PMOs grown alone (*control*) or cocultured with MDA PCa 2b cells. *Gapdh* was used as a loading control.

[5] Internet address: http://www.incyte.com.

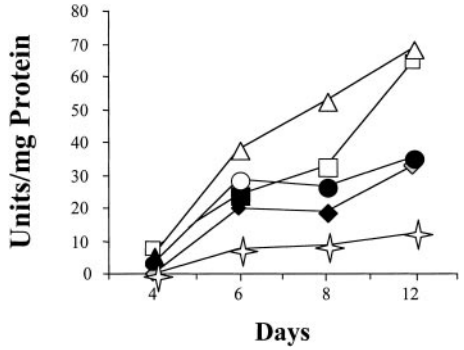

Fig. 4. Alkaline phosphatase activity in PMOs. PMOs were grown either alone (♦) or in coculture with MDA PCa 2a (□), MDA PCa 2b (△), LNCaP (●), and PC3 (✧). They were separated after 4 days of coculture. PMOs were subsequently grown in α-MEM plus 10% FBS until they reached confluence, and then they were placed in differentiation medium containing BMP-2. Alkaline phosphatase activity (units/mg protein) was measured at the indicated days using a Sigma Chemical Co. Diagnostics kit.

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-

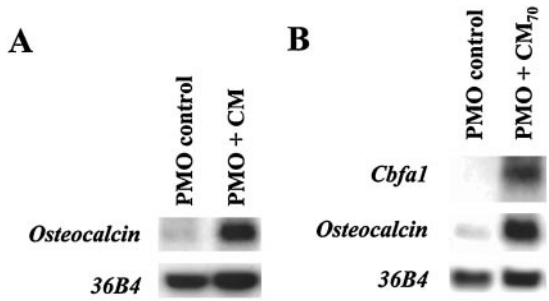

Fig. 6. A. Northern blot analysis of *Osteocalcin* expression in PMOs grown for 6 h in growth medium alone (*control*) or with CM obtained from MDA PCa 2b cells; these experiments were performed in serum-free conditions. *B*, Northern blot analysis of *Cbfa1* and *Osteocalcin* expression in PMOs grown for 12 h in growth medium alone (*control*) or in growth medium supplemented with CM_{70}. *36B4* was used as a loading control.

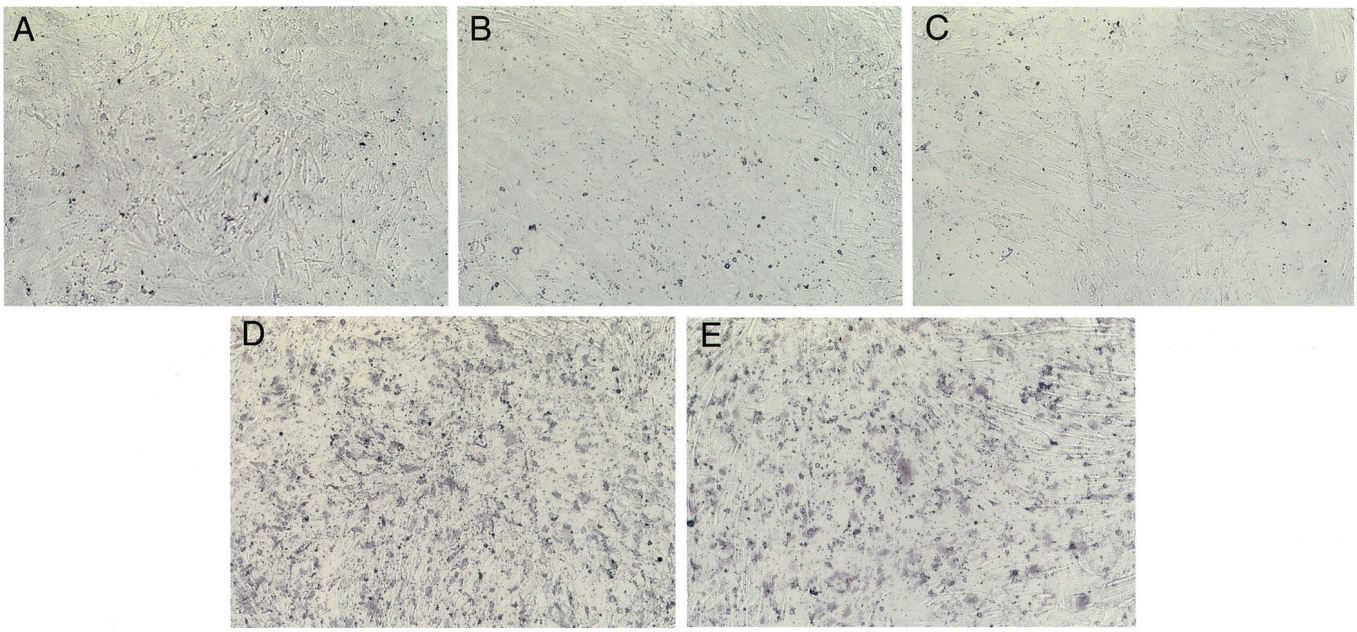

Fig. 5. To determine calcified matrix deposition, von Kossa staining was applied to PMOs after they had been grown in differentiation medium containing BMP-2 for 16 days. *A*, PMOs grown alone; *B*, PMOs grown with LNCaP cells; *C*, PMOs grown with PC3 cells; *D*, PMOs grown with MDA PCa 2b cells; *E*, PMOs grown with MDA PCa 2a cells.

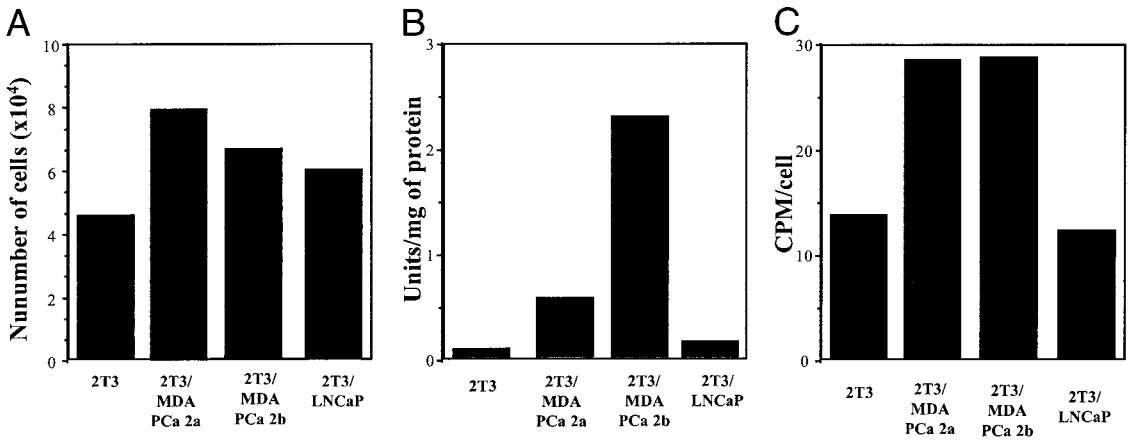

Fig. 7. Proliferation and markers of differentiation of the mouse osteoblast cells 2T3 grown alone (*2T3*) and after 4 days in coculture with MDA PCa 2a, MDA PCa 2b, and LNCaP cells. The cells were harvested and counted with a hematocytometer. The controls and each different coculture were assayed in triplicates. Alkaline phosphatase activity (units/mg protein) was measured with a Sigma Chemical Co. Diagnostics kit. Osteocalcin was evaluated by performing an immunoradiometric assay (Immunotopics, Inc).

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 Cbfa1 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 *Cbfa1* and *Osteocalcin* by PMOs. Fig. 6*A* 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 $\times$ CM_{70}, a substantial increase in *Cbfa1* and *Osteocalcin* expression was observed (Fig. 6*B*). These results indicate that soluble factors produced by MDA PCa 2b cells induce expression of *Cbfa1* and in turn *Osteocalcin*, a target of *Cbfa1*.

Coculturing with Prostate Cancer Cell Lines Induces Proliferation and Differentiation of Immortalized Murine 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 2T3 cells were cocultured with LNCaP cells.

Gene Expression Analysis. To study the molecular changes underlying the stimulation effect on osteoblast growth and differentiation induced by MDA PCa 2a and MDA PCa 2b cells, gene array analysis was performed. This analysis revealed that >7500 genes and expressed sequence tags showed measurable hybridization signals with both PMO controls and PMOs cocultured with MDA PCa 2b cells. The expression of 30 genes was increased ≥5-fold, and the expression of 44 genes was decreased ≥5-fold in PMOs after coculturing.[6] Among those genes, two families were of interest: extracellular matrix genes, including all procollagens tested, showed increase expression (Table 1), whereas transcripts associated with myoblast differentiation showed decreased expression (Table 2). Osteoblasts are bone-forming cells that, once terminally differentiated, produce most of the extracellular bone matrix, which is composed of collagenous and noncollagenous proteins. The main collagen produced by osteoblasts is procollagen type I, which accounts for 90% of the protein content of the bone matrix (27). Among the noncollagenous matrix proteins, bone sialoprotein and osteopontin are the most abundant. We performed a Northern blot analysis using RNA from another coculture experiment to confirm this modulation of gene expression. Fig. 8*A* illustrates that *Procollagen type I* was up-regulated in PMOs cocultured with MDA PCa 2b cells. In contrast, no up-regulation of *Procollagen type I* was observed when PMOs were grown in the presence of LNCaP or PC3 cells. Fig. 8*B* shows that PMOs grown in the presence of MDA PCa 2b cells up-regulated *Osteopontin* expression, confirming the results from the gene array study. Expression of *Myosin light chain 2* and *Myoglobin* was decreased in PMOs cocultured with MDA PCa 2b cells, compared with PMOs grown alone or in the presence of PC3 cells (Fig. 8, *C* and *D*). Taken together, these results indicate that MDA PCA 2b cells favor molecular events that led to osteoblast differentiation, while inhibiting myoblast differentiation.

MDA PCa 2b Cells Produced Osteoblastic Lesions *in Vivo*. All mice that had undergone intrabone injections with MDA PCa 2b cells had increased PSA levels. The median PSA blood levels were 4.5 ng/ml and 12 ng/ml at days 21 and 45, respectively, after the injections. 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 exhib-

Table 1 *Gene expression of extracellular matrix genes*

Balanced differential expression	Gene name[a]
6.0	*Mus musculus* mRNA for *collagen a1(V)*
9.5	*Procollagen, type XI, α 1*
9.5	*Procollagen, type III, α 1*
11.2	*Procollagen, type VI, α 1*
12.3	*Secreted phosphoprotein 1 (Osteopontin)*
18.9	*Procollagen, type V, α 2*
22.5	*Procollagen, type I, α 1*

[a] Reviewed in Ref. (27).

[6] Internet address: www.mdanderson.org/PMOGeneExpression.

Table 2 *Expression of genes related to myoblast differentiation*

Balanced differential expression	Gene name	Reference
−24.8	*Mus musculus myosin light chain 2*	Xu *et al.*, 2000 (47)
−10.8	*Muscle glycogen phosphorylase*	Froman *et al.*, 1998 (48)
−11.9	*Mus musculus* mRNA for stretch-regulated skeletal muscle protein (*Usmg4* gene)	Xu *et al.*, 2000 (47)
−10.7	*Myoglobin*	Garry *et al.*, 2000 (49)
−6.2	*Enolase 3, β* muscle	Keller *et al.*, 1992 (50)
−6.9	*Ryanodine* receptor 1, skeletal muscle	Ogawa *et al.*, 1999 (51)

ited 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 1×10^6 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 for at least 1 month developed osteoblastic lesions, and 12 (86%) rapidly developed osteolytic lesions (Fig. 9). Histopathological examination showed evidence of new bone formation when the bones had been injected with MDA PCa 2b. The left side of Fig. 10*B* shows MDA PCa 2b cells growing in the femoral shaft of the injected limb, surrounded by woven (immature) bone, which is typical of rapid bone formation. Surrounding the new bone is the lamellar (mature) bone. In the right side of Fig. 10*B*, higher magnification reveals the randomly deposited basket-weave pattern that is characteristic of new bone formation or bone remodeling (immature 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. 10*C*). The left side of Fig. 10*C* 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 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 transcriptor factor *Cbfa1*. Moreover, up-regulation of *Cbfa1* and *Osteocalcin* expression was also induced in PMOs by CM produced by MDA PCa 2b cells, suggesting that soluble factors produced by prostate cancer cells promote osteoblast differentiation and that *Cbfa1* mediates this effect. To our knowledge, this is the first *in vitro* model of bone metastasis from prostate cancer that recapitulates the osteoblastic phenotype typical of the disease. These results confirmed *in vivo* and at the molecular level, suggest that the pathophysiology of osteoblastic bone metastases from prostate cancer is related to an increase in bone formation rather than (or in addition to) a decrease in bone destruction.

The development, differentiation, and maturation of the osteoblast phenotype in primary cultures of rat calvaria osteoblasts are well characterized (20–22). Recent reports have also documented expression of markers of osteoblast differentiation in PMOs (23, 24). The primary cultures used in this study showed high levels of *Cbfa1* transcriptional activity and *Osteocalcin* expression, which indicate that PMOs are composed of osteoblasts and their precursors. MDA PCa 2a and MDA PCa 2b cells up-regulate markers of osteoblast differentiation, namely expression and secretion of *Osteocalcin*, secretion of alkaline phosphatase, and formation of bone matrix. We also detected increased expression of *Cbfa1* 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 extracellular 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-

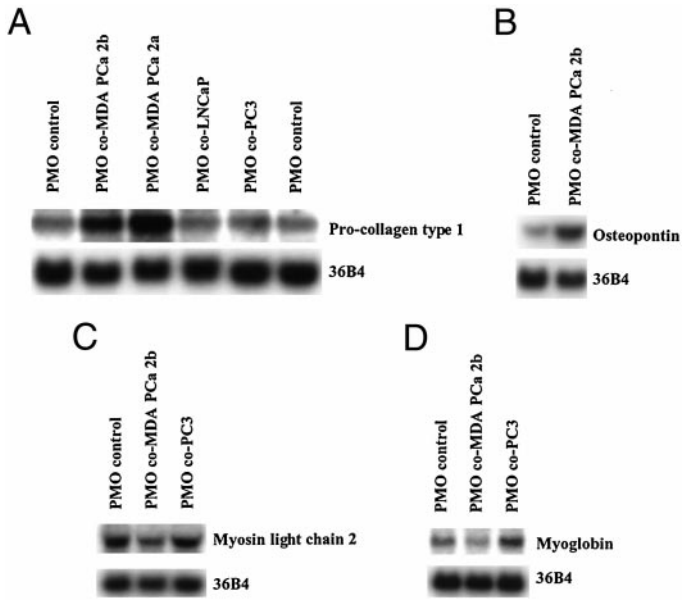

Fig. 8. Northern blot analysis of expressed genes in PMOs grown alone (*PMO control*) or after 4 days of coculturing with MDA PCa 2a, MDA PCa 2b, LNCaP, or PC3 cell lines. *A, Procollagen type I* expression; *B, Osteopontin* expression; *C, Myosin light chain 2* expression; *D, Myoglobin* expression. *36B4* was used as a loading control.

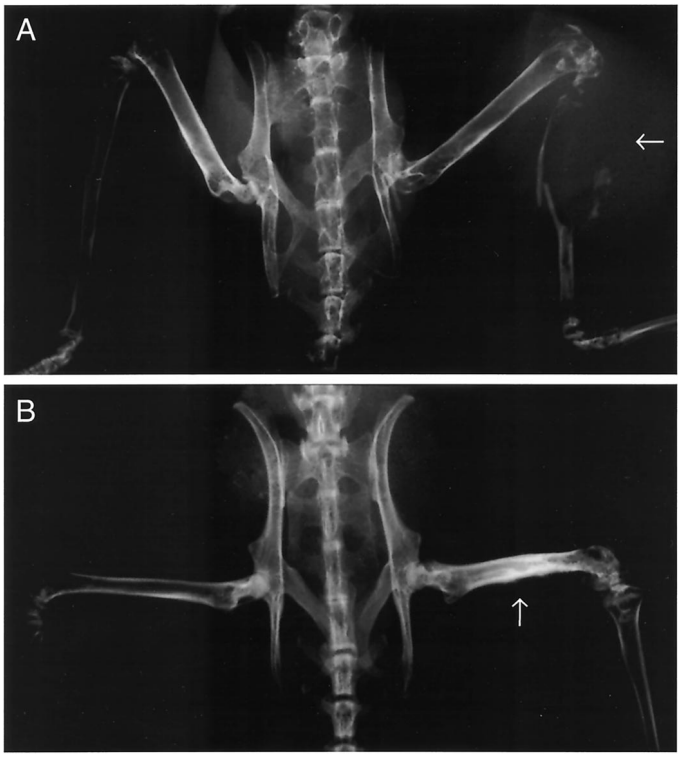

Fig. 9. X-ray imaging of mouse legs 6 weeks after intrabone injection of prostate cancer cells. *A*, thinning of cortical bone after injection of PC3 cell, suggesting increased bone resorption. *B*, increased bone density after injection of MDA PCa 2b cell.

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 cell lines. 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.

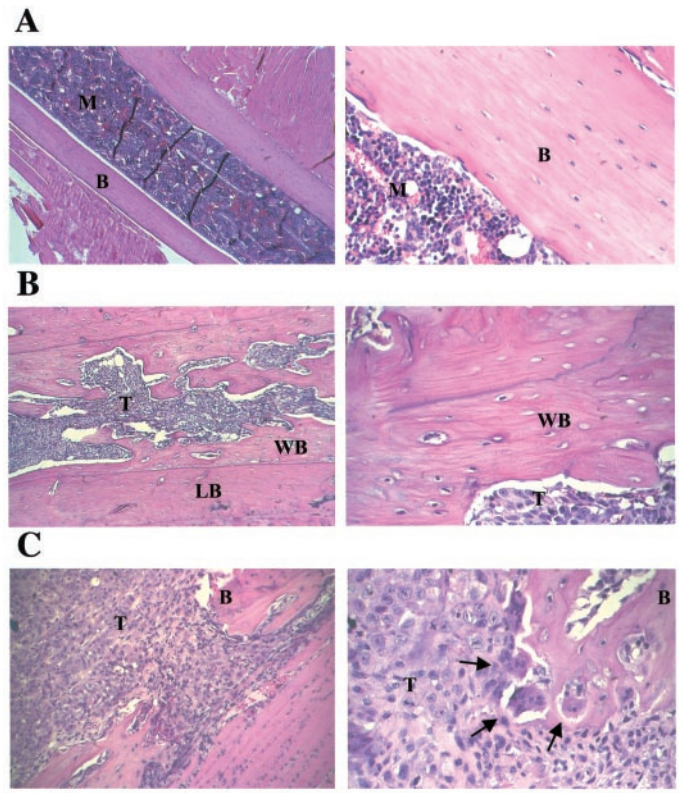

Fig. 10. *A*, longitudinal section of mouse femur stained with H&E (*left*: ×40, *right*: ×200). *Right panel*, normal bone with lamellar pattern, which is seen after normal bone formation (*i.e.*, old or mature bone). *B*, mouse femur after intrabone injection of MDA PCa 2b cells showing microscopic evidence of new bone formation. *C*, histological evidence of osteolytic lesions in the femur of a mouse injected with PC3 cells. Osteoclasts (*arrows*) are obvious at the tumor bone interface. *M*, bone marrow; *B*, cortical bone; *T*, tumor; *WB*, woven (immature) bone; *LB*, lamellar (mature) bone.

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Prostate Cancer Cells Induce Osteoblast Differentiation through a *Cbfa1*-dependent Pathway

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