Osteoblast-Derived Factors Induce an Expression Signature that Identifies Prostate Cancer Metastasis and Hormonal Progression


Abstract

Identification of gene expression signatures associated with metastases provides a tool to discern mechanisms and potential therapeutic targets and may lead toward a molecular classification system in pathology. Prostate cancer (CaP) frequently metastasizes to the bone to form osteoblastic lesions. Correlative clinical data and in vitro evidence have led to the hypothesis that osteoblast-derived factors promote hormonal progression of CaP cells. Here, the gene expression signature of CaP exposed to osteoblast-derived factors was identified. This signature included known androgen-regulated genes, oncogenes, tumor suppressors, and genes whose products are involved in apoptosis and cell cycle. A comparative functional genomic approach involved the application of this responsive gene expression signature to clinical samples of human CaP, melanomas, and oral cancers. Cluster analysis revealed that this gene expression signature had specificity for CaP and could resolve clinical specimens according to stage (benign, localized, and metastatic) and androgen sensitivity with an accuracy of 100% and 80%, respectively. Together, these results suggest that factors derived from osteoblasts induce a more advanced phenotype of CaP and promotes hormonal progression. [Cancer Res 2009;69(8):3433–42]

Introduction

Prostate cancer (CaP) metastasizes to the bone to form predominantly osteoblastic lesions (1). The mechanism underlying the propensity of CaP to metastasize to the bone is currently unknown. The only effective treatment for CaP patients with metastatic disease is androgen ablation. However, in spite of an initial clinical response to the androgen ablation therapy, there is an ultimate development of the castration-recurrent disease, which is indicated by an increasing titer of serum prostate-specific antigen (PSA). There is no known cure for castration-recurrent CaP, and the mechanisms associated with hormonal progression remain elusive.

Androgen receptor (AR) is suspected to be important in the mechanism involved in hormonal progression of CaP (2). AR can be activated in an androgen-independent manner by a number of factors, including those derived from human osteoblasts (3). Thus, bone-derived factors may facilitate survival and progression of CaP to castration recurrence by cross-talk between AR and alternative signal transduction pathways (3).

To explore the mechanism by which the bone microenvironment may promote survival and progression of CaP, we obtained a gene expression signature of CaP cells exposed to osteoblast-derived factors. Application of this signature to clinical samples of CaP distinguished clinical metastasis and hormonal progression of CaP. This gene expression signature potentially reveals genes involved in the advanced phenotype of CaP that enable a growth advantage in the distant site of colonization.

Materials and Methods

Cell culture and treatment. LNCaP, 22RV1, and RKO human colon cancer cells were treated in serum-free and phenol red–free media. Conditioned media from osteoblast-like cells (OCM) and fibroblasts (FCM) were prepared (3, 4). Briefly, bone chips from femoral head trabecular explants were cultured in MEM containing 20% fetal bovine serum. Outgrown osteoblast-like cells were characterized using the reverse transcription–PCR (RT-PCR) for expression of the osteoblast markers type I procollagen, alkaline phosphatase, and osteocalcin (5). OCM was collected after culturing osteoblast-like cells for 48 h in serum-free MEM. B26, B33, and B44 refer to OCM prepared from bone samples from patients 26, 33, and 44, respectively.

Cell proliferation analysis. LNCaP, 22RV1, and RKO cells in 96-well Falcon Primaria tissue culture plates (60% confluence) were treated with 0.00000385% v/v ethanol (vehicle control), 0.1 nmol/L R1881, OCM (50% v/v), or FCM (50% v/v). After 5 d, cell proliferation was assessed using a BrdUrd ELISA kit (Roche Diagnostics).

Flow cytometry. LNCaP cells were treated with ethanol, 0.1 nmol/L R1881, or OCM (50% v/v) in serum-free and phenol red–free RPMI 1640 (sRPMI 1640). At 48 h later, cells were treated with 100 μL of 1 μg/mL BrdUrd for 1 h at 37°C. BrdUrd staining was performed (BD Biosciences), and cells were analyzed by FACSCalibur (BD Biosciences) and Flow-Jo software (Tree Star, Inc.).

Fluorescent microscopy. LNCaP cells (5 × 104) were treated with ethanol, 0.1 nmol/L R1881, or OCM (50% v/v) in sRPMI 1640. After 3 d, cells were stained using the ApopTag Fluorescein apoptosis detection kit (Chemicon International), counterstained with 4,6-diamidino-2-phenylindole, and examined using an Axioplan-2 fluorescence microscope (Zeiss).

Animal models. The LNCaP hollow fiber model was performed (6, 7) with modifications. Briefly, hollow fibers containing LNCaP cells alone or cocultured with osteoblasts (1 × 104 each) were s.c. implanted into male nude mice. All procedures were performed in compliance with regulations under an appropriate animal license issued by the University of British Columbia. Blood samples were obtained from mice weekly, and serum PSA was measured using an ELISA kit (Abbott IMX).
Affymetrix GeneChip analysis. Cells were serum starved for 48 h and subsequently treated with OCM or MEM. Total RNA from three independent experiments was extracted from cells using Trizol (Invitrogen). HGU133plus2/HGU133A Affymetrix GeneChips were used for microarray analyses (Affymetrix). Hybridizations were completed independently for each condition using three biological replicates.

Expression profile analysis. Comparative analysis between expression profiles for Affymetrix experiments used GeneSpring software version 7.2 (Silicon Genetics). The expression profiles from three independent experiments were compared using the two-sample Student’s t test (parametric test, assume variances equal) to identify genes that were differentially expressed. Affymetrix data sets of clinical CaP were downloaded from PubMed GEO (GDS1439, GSE6919, and GDS1390). For sample clustering, Pearson correlation was applied to measure the similarity of the expression pattern between different samples according to the expression profile of certain gene lists. For principle component analysis, principle component scores were calculated according to the standard correlation between each condition vector and each principle component vector. For class prediction analysis, classification was generated by the “support vector machines” algorithm, using the training set “OCM-treated experiment” and the test set “CaP metastasis clinical samples” or “CaP hormonal progression samples” for the parameter “treatment type.” All of the 40 genes from our signature profile were used in the analysis. The OCM margin from individual clinical samples was calculated to represent the likelihood of being exposed to OCM.

RT-PCR on clinical samples. Clinical bone metastases and lymph node metastases were obtained from the Prostate Cancer Rapid Autopsy Program, University of Washington, by coauthor R.LV. Real-time RT-PCR used gene-specific primers (Supplementary Table S1) and SYBR green supermix (Invitrogen) in an ABI 7900 machine (Applied Biosystems) with cycling conditions at 95°C for 2 min followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. Glyceraldehyde-3-phosphate dehydrogenase–normalized expression levels of individual genes were input into GeneSpring software (v7.2). For sample clustering, Pearson correlation was applied to measure the similarity of the expression pattern between different samples according to the expression profile of these 10 genes.

Protein analysis. LNCaP cells incubated in sfRPMI for 24 h, after which cells were treated as in the figure legends. Immunoblot analysis used cells lysed in SDS-PAGE sample buffer. Signal transducers and activators of transcription 3 (STAT3) immunoprecipitation used anti–total STAT3 (Cell Signal). Blots were probed with antibodies against phosphorylated or total E2F1/2, Cell Signal, ErBb2, or AKT (R&D Systems).

Results

Osteoblast-derived factors promote survival and proliferation of CaP cells. To investigate the mechanism(s) underlying the progression of CaP in response to osteoblast-derived factors in the absence of androgen and serum, we used OCM and examined changes in proliferation of LNCaP and 22RV1 cells by BrdUrd incorporation. Osteoblast-like cells from different bone donors (e.g., B45, B66, and B70) were used to obtain OCM. The proliferation of both CaP cell lines increased in the absence of androgen when treated with OCM (final concentration, 50%) compared with the untreated control (Fig. 1A). Androgen (0.1 nmol/L, R1881) increased the proliferation of both CaP cell lines, as expected (positive control; refs. 8, 9). Consistent with previous findings, there was no increase in proliferation of CaP cells in response to FCM (10) nor was there any significant effect on proliferation of the colon cancer cell line (RKO) in response to OCM. These data support that OCM increases proliferation of CaP cells and that this effect is not broadly observed for all cancers.

The effect of OCM treatment on the different phases of the cell cycle was analyzed using flow cytometry. R1881 (positive control) induced a 2-fold increase ( 42%, P < 0.001) in the percentage of cells in the S phase compared with the control group that had ~20% of the population in the S phase (Fig. 1B). In the absence of androgen, OCM-treated samples had a relatively high percentage of cells in S phase (~34%, P < 0.005) compared with the control. These data indicate that OCM increased the proliferation of CaP cells in the absence of androgen.

To evaluate the role of OCM on the survival of LNCaP cells, DNA fragmentation nicks associated with apoptotic cells were labeled and visualized using immunofluorescence. In the absence of serum and androgen, ~10% of the total population of LNCaP cells had positive staining for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL; Fig. 1C). Androgen decreased staining for TUNEL to ~4% (P < 0.001), whereas OCM-treated cells showed even further decrease in TUNEL staining to ~2% (P < 0.001). Collectively, these results suggest that osteoblast-derived factors induce androgen-independent proliferation and survival in CaP cells.

Osteoblasts promote hormonal progression of CaP in vivo. The effect of osteoblasts on the hormonal progression of CaP in vivo was investigated using the LNCaP hollow fiber model that we previously developed. LNCaP cells grown in hollow fibers progress to the castration-recurrent stage (6). This model was modified by mixing an equal density of LNCaP cells with osteoblasts within fibers that were then implanted s.c. into mice (Fig. 2A). Animals were also implanted with fibers containing only LNCaP cells (Fig. 2B) or a mixture of LNCaP cells and fibroblasts (HS68). Animals were castrated, and levels of serum PSA were monitored to indicate hormonal progression and increased tumor burden (11). Castration resulted in a 70% drop in PSA in mice bearing LNCaP cells grown with osteoblasts compared with 90% decrease in PSA levels observed after castration of mice bearing solely LNCaP cells (Fig. 2C). LNCaP cells mixed with fibroblasts showed no significant difference from solely LNCaP cells (data not shown). These data were further supported using a modification of the s.c. xenograft model (ref. 4; Supplementary Fig. S1).

Together these results (a) mimic the clinical scenario whereby patients with bone lesions tend to have a higher PSA nadir than those patients with soft tissue involvement and (b) suggest that osteoblasts promote castration-recurrent CaP.

The gene expression signature of CaP cells induced by osteoblast-derived factors. The genome-wide expression profile of LNCaP cells in response to osteoblast-derived factors was identified using serum-free and androgen-free conditions. Cells were exposed to OCM for 48 hours before total RNA was extracted and analyzed using Affymetrix GeneChips to determine the global gene expression signature. Significance analysis of microarrays (12) was applied to identify genes differentially expressed across various conditions. Using a two-way ANOVA (P ≤ 0.05) and 1.5-fold change cutoff, 181 genes were differentially expressed across various conditions (Supplementary Table S2). Of these genes, there were 40 with known function in cancer, apoptosis, and cell cycle (Supplementary Fig. S2; Supplementary Table S3). Levels of expression of all 40 genes were validated by RT-PCR (data not shown). Oncogenes RAB3B, BIRC5 (survivin), and STK6 (AURKA) had increased expression, whereas expression of a candidate tumor suppressor gene, C6orf210 (DLP1), was decreased. Some regulators of apoptosis were also differentially expressed in response to OCM and included up-regulation of apoptosis inhibitors MALT1, ESP1L, and PAR1 and down-regulation of apoptosis mediators F2R, BCAP29, CRADD, and STK3. Consistent with the increased
proliferation in response to OCM, a number of cell cycle–related genes were differentially expressed in response to OCM. The genes known to function in particular phases of the cell cycle or checkpoints are shown (Supplementary Fig. S2D). The most striking changes were the increased expression of a group of cyclins and CDCs (CCNA2, CCNB1, CCNB2, CCNE2, CDC2, CDC6, CDC7, CDC20, and CDC25C), with an exception of CDC16, which encodes a component protein of the APC complex, a cyclin degradation system. Other genes differentially expressed in response to OCM included increased expression of vascular endothelial growth factor (VEGF) and cell cycle genes GTSE1, HCAP-G, AURKB, BUB1, BIB1B, MCM5, PLK1, CDKN3, ASNS, CHEK1, DEPDC1, ESP1L1, SM4CL1, and STK6 and decreased expression of F2R, HECA, and ACPP. These global changes in the transcriptome of CaP cells in response to OCM suggest that factors derived from osteoblasts modify the expression of cell cycle–related genes aiding in the proliferation of CaP cells in the absence of androgen. These 40 genes comprised a gene expression signature for CaP cells exposed to osteoblast-derived factors in the absence of both androgen and serum.

**Prosurvival and proliferative signaling pathways are activated in LNCaP cells that are exposed to osteoblast-derived factors.** AR is activated by alternative pathways in CaP in the absence of androgen by osteoblast-derived factors (3). We measured the levels of six factors suspected to be involved in CaP metastases in the OCM by ELISA. Only the levels of interleukin-6 (IL-6) and VEGF were consistently higher in OCM preparations compared with the levels measured in controls (Supplementary Fig. S3). Neutralization of IL-6 blocked proliferation in several preparations of OCM (3). Consistent with this, IL-6 neutralization consistently blocked OCM-induced expression of PSA, CCNB1, and CCNA2 in all three OCM preparations analyzed, whereas the expression of CCNB2, AURKB, and CDC2 was blocked in two of three OCM preparations (Supplementary Table S4). Thus, IL-6 is one of the major factors present in OCM that increases the proliferative phenotype of CaP in the absence of androgens.

IL-6 can activate the Janus-activated kinase (JAK)/STAT3, mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase, and ErbB2 pathways, all of which have been implicated in hormonal progression of CaP (ref. 2; Supplementary Fig. S4 shows schematic representation of these pathways). Of particular interest, STAT3 is implicated in promoting the metastatic potential of CaP (13), and its activity, or markers thereof, is often found to be elevated in clinical CaP samples (14, 15). ErbB2 activation leads to enhancement of AR activity in the absence of ligand (16) and its overexpression has been observed at least in a subset of hormone
refractory CaP patients (17–19). The status of IL-6 pathways in OCM-treated LNCaP cells was queried by measuring the phosphorylation of critical proteins by Western blot analyses. STAT3 tyrosine and serine phosphorylation levels were readily detectable upon OCM treatment but not in cells treated with the control FCM or conditioned media from LNCaP (Fig. 3A, I and II). JAK2 activation may be upstream to these effects because its inhibitor, AG490, blocked STAT3 phosphorylation (Fig. 3A, III). Preincubation with IL-6 neutralizing antibody, but not with the isotype IgG control, blocked OCM-inducible STAT3 phosphorylation (Fig. 3A, IV). Consistent with STAT3 involvement, known gene targets of STAT3, including PIM1, BCL-2, VEGF, and BIRC5/survivin, were up-regulated at the mRNA level in OCM-treated LNCaP cells (Supplementary Table S2; data not shown). OCM increases the PSA promoter reporter activity (3), and this was significantly decreased upon pretreatment with nontoxic concentration of AG490 (data not shown). These results collectively suggest that IL-6 is the component in the OCM that activates STAT3 and may lead to increased AR transcriptional activity and/or transcriptional regulation of STAT3 target genes.

The phosphorylation of ERK1/2, ErbB2, and AKT was also increased in LNCaP cells upon treatment with OCM (Fig. 3B and C). IL-6 neutralization did not completely block ERK phosphorylation nor was there any detectable effect on the activation of ErbB2 (Supplementary Fig. S5A and B). OCM-inducible ERK activation was blocked by the ErbB2 inhibitor AG879 and, as expected, by the MAPK/ERK kinase inhibitor, U0126 (Fig. 3D). Thus, factors other than IL-6 likely mediate the OCM-inducible activation of ErbB2 and the downstream MAPK activity, and this may contribute to enhanced growth of CaP. Thus, although the IL-6/JAK/STAT3 axis might be largely responsible for the effect of OCM on androgen-independent growth of LNCaP cells, IL-6–independent mechanisms may also be involved.

Osteoblast-derived factors stimulate the androgen axis in CaP cells. Osteoblast-derived factors induce the expression of PSA and the activities of reporter gene constructs containing AREs by a mechanism dependent on AR (3). Thus, in the absence of androgen, osteoblasts may release factors that activate AR in CaP cells, which may promote castration-recurrent disease. To characterize the status of the AR pathway in the OCM-treated cells, a hierarchical two-dimensional clustering algorithm based on similarity of expression patterns was applied to the 1,092 genes previously identified to be differentially expressed in LNCaP cells treated with R1881 (7) and OCM (represented on each column). Comparison of expression profiles showed a strong tendency to subgroup the samples with respect to the treatments (Fig. 4). This suggests that distinct and consistent differences in expression profiles occurred with each treatment. Of particular interest, hierarchical cluster analysis revealed that the expression patterns of androgen-regulated genes for the OCM-treated samples were more similar to that of the R1881-treated samples, whereas the control samples from these two experiments were clustered together. Applying the Venn diagram, 19 genes were identified as common targets for changes in expression in response to both androgen and OCM (Supplementary Table S5). The levels of expression of the majority of these genes were increased (ATAD2, BM039, INSIG1, KLK2, KLK3, MALT1, PDE9A, PDIR, RAB3B, TNFRSF10B, UAP1, and VEGF), whereas the levels of expression of OSR2, PIK3R1, and 215441_at decreased by both androgen and OCM. Only expression of APPBP2, BRE, and SOCS2 was regulated in opposite directions.

Figure 2. Osteoblasts promote in vivo hormonal progression of CaP. H&E staining of LNCaP cells cocultured with osteoblast-like cells (OB; A) or without osteoblast-like cells (B) in hollow fibers that were implanted in mice. C, changes in levels of serum PSA from mice bearing fibers containing solely LNCaP cells (n = 12) or fibers with LNCaP and osteoblast-like cells (LNCaP + OB; n = 12) before and after castration (Cx). Points, mean; bars, SE. Student's t test: *, P < 0.05.
These data support that a subset of androgen-regulated genes become reexpressed in the absence of androgen by factors derived from osteoblasts, thereby potentially circumventing the need for androgen to enhance survival and growth of CaP cells (3). Expression levels of this subset of genes may be prognostic for the development of advanced, castration-recurrent CaP.

**Gene expression signature induced by osteoblast-derived factors identifies clinical metastasis of CaP.** Bone metastasis is the major cause of CaP-related morbidity and mortality (20). CaP cells exposed to osteoblast-derived factors may reveal genes involved in tumor formation and growth at the metastatic site. Therefore, the gene expression signature obtained from LNCaP cells treated with OCM may be common to that observed in clinical metastases. To test this hypothesis, we applied our gene expression signature to clinical samples of CaP from various stages, which included benign (n = 6), localized (n = 7), and metastatic (n = 6; ref. 21), using a hierarchical two-dimensional clustering algorithm based on similarity of expression patterns. Comparison of expression profiles showed a strong tendency to subgroup the samples with respect to tumor stage (Fig. 5A), and the metastasis samples were more distinct from other stages. Consistent with our hypothesis, LNCaP cells that were treated with OCM clustered with the clinical metastasis samples.

Unsupervised principal component analysis based on the largest three principal components revealed separate clustering of the different stages of tumor samples along principal component 1 (71.87% variance; Fig. 5A). This indicates that the presence of the majority of these genes (~29, if the variance was equal for all 40 genes) can distinguish the metastatic tumor samples from the benign prostate tissue and localized CaP. These data also showed separate clustering of the benign samples from the CaP samples (including localized and metastatic) along the same component.

We further applied the class prediction analysis to evaluate if our gene expression signature could identify clinical CaP metastasis. The OCM margin from individual clinical samples was calculated to represent the likelihood of exposure to osteoblast-derived factors. All samples of CaP metastasis had a positive OCM margin, whereas all the localized CaP samples and benign tissues had a negative OCM margin. Our gene expression signature was able to distinguish metastatic CaP from localized CaP with 100% accuracy (P = 1.21714e−05), as well as localized CaP from benign tissues (P = 0.04; Fig. 5B, II). To confirm the value of this signature, we applied a bigger sample pool of 18 benign prostate tissues, 63 subbenign samples from the tissues beside the primary tumor, 61 primary CaP, and 25 samples of metastatic CaP (22, 23). Similar to the smaller sample pool, unsupervised principal component analysis revealed separate clustering of the different stages of tumor samples (Fig. 5C, I). For the class prediction analysis, most of the CaP metastasis samples (22 of 25) had a positive OCM margin whereas most of the localized CaP samples (50 of 61), the subbenign samples (61 of 63), and the benign prostate tissues (16 of 18) had a negative OCM margin (Fig. 5C, II). Our gene expression signature could resolve metastatic CaP from localized CaP with 89% accuracy (P = 1.66e−13) and localized CaP from subbenign tissues (P = 0.017). Our gene expression signature could not distinguish the metastasis samples from the primary samples of hypopharyngael (GDS1070) and oral cancers (GDS1062; data not shown) but did have prediction accuracy of ~84% for melanoma metastasis (GSE8401; Supplementary Fig. S6).

To aid in revealing how factors derived from osteoblasts may promote the metastatic phenotype in CaP, we more closely

![Figure 3](https://example.com/fig3.png)

**Figure 3.** OCM-inducible signaling pathways. A, I and II, levels of tyrosine phosphorylation (pTyr STAT3), serine phosphorylation (pSer STAT3), and total (tSTAT3) STAT3 in LNCaP cells treated with 50% of OCM, 50 ng/mL IL-6 (positive control), and negative controls of FCM and conditioned media from LNCaP (LCM). III and IV, cells were pretreated for 30 min with the JAK2 inhibitor AG490 (50 μmol/L). IL-6 was blocked by pretreating the OCM with IL-6 neutralizing antibody (Anti-IL6; 10 μg/mL). IgG served as an isotype control. B, phosphorylated (pErk1/2) and total (tErk1/2) ERK1/2 levels. C, I, phosphorylated erbB2 (pErbB2) and phosphorylated AKT (pAKT) levels. II, cells were pretreated for 30 min with the ErbB2 inhibitor AG879 (20 μmol/L). CRP, a cross-reactive protein (loading control). D, effects of kinase inhibitors on OCM-induced ERK1/2 phosphorylation. Cells were pretreated with inhibitors or vehicle (DMSO) for 30 min before treatment with 50% OCM.
examined the expression of some of the individual cancer/apoptosis/cell cycle–related genes in CaP metastasis. Both OCM-regulated oncogenes, BIRC5 and STK6, were up-regulated in samples of metastases (Supplementary Fig. S7A). Apoptosis mediators, CRADD and BCAP29, were down-regulated, whereas apoptosis inhibitor EPL1 was up-regulated. All of the OCM-regulated cyclins (CCNA2, CCNB1, CCNB2, and CCNE2) and most of the OCM-regulated CDcs (CDC2, CDC6, CDC20, and CDC25C) were up-regulated in metastases. Negative cell cycle regulator (ACPP) was down-regulated, whereas VEGF and cell cycle mediators (ASNS, AURKB, BUB1, BUB1B, CDKN3, CHEK1, DEPDC1, ESPL1, GTSE1, HCAP-G, MCM5, PLK1, SM4L1, STK6) were up-regulated in metastases (P ≤ 0.05). The expression of these genes in metastasis had the same trend as in the OCM-treated samples, with exception of C6orf210, F2R, HECA, and MALT1.

To test if these OCM-regulated genes could distinguish between sites of metastases, we compared clinical castration-recurrent samples of bone metastasis (n = 6) to lymph node metastasis (n = 6). Comparison of expression profiles showed a strong tendency to subgroup the samples with respect to site of metastasis with two lymph node metastasis samples “misgrouped” with the bone metastasis samples (Fig. 5D).

**Gene expression signature induced by osteoblast-derived factors identifies castration-recurrent CaP.** Osteoblast-derived factors can enhance androgen-independent proliferation and reduce apoptosis of CaP cells (Figs. 1 and 2). Thus, changes in the gene expression profile of CaP cells in response to these factors may contribute to hormonal progression. To test this hypothesis, we applied our gene expression signature to clinical samples of both androgen-dependent (n = 10) and castration-recurrent (androgen-independent; n = 10) lesions (24). Upon comparison of expression profiles, samples seemed to subgroup according to their dependency upon androgen with the exceptions of AD-GSM45854 and AI-GSM45863 (Fig. 6A). Unsupervised principal component analysis revealed briefly separate clustering of the different stages of tumor samples (Fig. 6B). Class prediction analysis revealed that 70% of the castration-recurrent samples (7 of 10) have a positive OCM margin whereas 90% of the androgen-dependent samples (9 of 10) have a negative OCM margin (Fig. 6C). This gene expression signature was able to distinguish the castration-recurrent clinical samples with an accuracy of 80% (P = 0.0056). Together, these analyses suggest that factors derived from osteoblasts induced a relatively distinct transcriptional signature that may contribute to the castration-recurrent phenotype. Genes overlapping between the gene expression signatures for OCM and castration-recurrent clinical tissue included cell cycle regulators CCNB1, CDC20, CHEK1, ESPL1, HCAP-G, SM4L1, and STK3 (up-regulated in castration-recurrent samples) and a candidate tumor suppressor gene ACPP (down-regulated in castration-recurrent samples; Supplementary Fig. S7B).

**Discussion**

CaP frequently metastasizes to the bone, which is associated with hormonal progression (25). Here, the effect of exposure of CaP cells to osteoblast-derived factors yielded the following observations: (a) OCM promotes the survival and proliferation of CaP cells, (b) in vivo osteoblasts promote hormonal progression of CaP, (c) the gene expression profile of CaP cells in response to OCM was identified, (d) OCM induced the transcriptional program in CaP cells that partially overlapped with the transcriptional program for androgen, (e) a gene expression signature induced by OCM could distinguish between clinical metastasis of CaP and other stages of the disease, and (f) this gene expression signature distinguished castration-recurrent from androgen-dependent clinical tissue.

CaP is generally more aggressive when it has metastasized to distant sites, especially in the bone (26). Consistent with the clinical observations of CaP patients harboring osteoblastic lesions who are receiving androgen ablation therapies, we showed that OCM induces androgen-independent proliferation of CaP cells maintained in cell culture and in vivo in castrated hosts (3, 8, 4, 9). In agreement with colon cancers rarely resulting in bone metastasis (27), OCM did not significantly increase the proliferation of RKO colon cancer cells. Thus, osteoblast-derived factors do not broadly increase proliferation of all cancer cells and may be relatively specific for CaP. Such observations are consistent with the high frequency of CaP metastasis to the bone to form osteoblastic lesions.
Identification of changes in gene expression of CaP cells in response to OCM may reveal mechanisms for phenotypic changes in osseous lesions and tissue tropism. Here, we provide the first report that investigates changes in the global transcription program of CaP in response to OCM in the absence of androgens. There were 181 genes that were significantly differentially expressed in CaP cells in response to OCM (OCM181). The OCM181 signature identified metastatic samples in both small (Supplementary Fig. S8 A and B) and large sample pools (Supplementary Fig. S8 C and D) but did not perform as well as the gene expression signature based upon the 40 genes (the accuracy of 95% versus 100% in smaller sample pool and 83% versus 89% in bigger sample pool; Fig. 5). In this set of 40 genes, the most striking finding was the enrichment of cell cycle–related genes. All cyclins and most of the CDC genes identified showed increased expression in response to OCM. Other important genes, such as VEGF, AURKB, MCM5, and PLK1, were all up-regulated, whereas ACPP, which mediates vitamin D–induced cell growth arrest (28), was down-regulated in response to OCM. Together, these combined changes in the gene expression profile may lead to the survival and proliferation of CaP and contribute to hormonal progression to the castration-recurrent phenotype.

AR is suspected in the mechanism of hormonal progression of CaP and is activated by osteoblast-derived factors to increase expression of PSA and reporter gene constructs in the absence of androgen (3). Here, a comprehensive analysis of changes in gene expression.

**Figure 5.** OCM gene expression signature can distinguish various stages of CaP. Affymetrix data sets of clinical CaP were downloaded from PubMed GEO (GDS1439 and GSE6919). A total of 40 OCM regulated genes from Supplementary Table S2 were applied as the gene signature. **A,** sample clustering to measure the similarity of the expression pattern between different samples from GDS1439 according to the gene expression signature. **B,** principle component analysis distinctly clustered the clinical samples from GDS1439 into three groups which closely correlate to the three different stages (benign prostate tissue and localized and metastatic CaP). **C,** class prediction analysis applying the gene expression signature provided each clinical sample from GDS1439 with an individual OCM margin, which represents the likelihood of exposure to OCM. Student’s t test was applied to calculate the P value for the difference between groups. **D,** sample clustering to measure the similarity of the Q-PCR measured expression pattern between clinical samples from different metastasis sites according to the expression of a subset of genes in the signature. LM, lymph node metastasis.
expression in response to OCM supports activation of AR pathway in the absence of androgens. Nineteen genes were differentially expressed in response to both androgens and in the absence of androgens by OCM (genes are summarized in Supplementary Table S5). This number was higher than expected by chance (181 / 1,092 = 3.66; 181 and 1,092 are the numbers of OCM or androgen-responsive genes, respectively; 54,000 is the total number of genes represented on the Affymetrix GeneChip U133plus2.0). Expression of androgen-responsive genes provides clinical evidence supporting the importance of AR in metastasis and hormonal...
progression. AR may be an important quality of CaP that facilitates colonial outgrowth in the bone.

After the lymph nodes, bone is often the exclusive site of metastasis of CaP and is associated with poor prognosis and hormonal progression (29). Based upon these observations combined with the data presented here, factors derived either solely from osteoblasts or combined with those from the metastatic niche must provide advantages for CaP cells to survive, colonize, and escape androgen ablation therapy to proliferate in the absence of testicular testosterone. Support that osteoblast-derived factors play a major role in these mechanisms can be drawn from the ability to identify clinical samples of metastasis (100% accuracy, \( P = 1.21714 \times 10^{-05} \)) and hormonal progression (80% accuracy, \( P = 0.04 \)) using the gene expression signature of 40 genes from CaP cells exposed to OCM. This gene expression signature was more specific than the OCM181 signature for the identification of advanced CaP that lacked the power to identify castration-recurrent samples (\( P = 0.32 \), Supplementary Fig. S9) and showed specificity for the bone when comparing bone metastases to other sites of metastasis (Fig. 5D). These data imply the importance of key common genes in the transcriptional program of clinical samples of metastases and castration-recurrent disease to that obtained in CaP cells exposed to OCM in the absence of androgens. Similarities of differential gene expression between these clinical samples using our gene expression signature revealed 31 of 40 genes that were significantly differentially expressed in the CaP metastasis samples whereas only eight genes were differentially expressed in the castration-recurrent samples.

This gene expression signature surprisingly identified clinical samples of metastases from localized lesions for melanoma, but not for oral and hypopharyngeal cancers. Together, these data suggest specificity of this gene expression signature for cancers that metastasize to the bone because the bone is also a major site of metastasis for melanoma (30). The specificity of the gene expression signature of cells exposed to OCM to be common for cancers that metastasize to the bone supports the Paget hypothesis that metastasis can only form when the seed (cancer) and soil are compatible (31). Cancers of different origin, such as CaP and melanoma, may share some similar characteristics for the selection of soil (bone), while being clearly distinct from other types of tumors, such as oral and hypopharyngeal cancers. Identification of the transcriptional program of the seed in response to selective pressures from the soil that shapes its malignant phenotype will ultimately reveal new therapeutic targets and strategies to reduce morbidity and mortality from cancer.

Recently, a gene expression signature from early-stage cancer was associated with metastases and poor clinical outcome in a variety of solid tumors (32). The generic signature shared in all types of solid tumors identified by Ramaswamy may reflect how the tumor cells escape from the original sites to enter the circulation. Whereas this is an important aspect for metastases, it does not and cannot address all steps required for successful colonization of cancer cells at distant sites to form lethal metastatic disease. CaP patients may have significant numbers of disseminated tumor cells that remain dormant for many years and will not result in the formation of macrometastases. Colonization of cancer cells is generally accepted as the rate-limiting determinant in metastases. The work shown here stresses this important aspect of how the escaped tumor cells may acquire an aggressive phenotype to obtain an advantage in the distant sites of colonization.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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