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Osteoblast-derived factors induce an expression signature that identifies prostate cancer metastasis and hormonal progression

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Abstract

Identification of gene expression signatures associated with metastases provides a tool to discern mechanisms and potential therapeutic targets and may lead towards a molecular classification system in pathology. Prostate cancer 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 prostate cancer cells. Here the gene expression signature of prostate cancer 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 the cell cycle. A comparative functional genomic approach involved the application of this responsive gene expression signature to clinical samples of human prostate cancer, melanomas, and oral cancers. Cluster analysis revealed that this gene expression signature had specificity for prostate cancer 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 prostate cancer and promotes hormonal progression.

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 ultimate development of 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.

The AR is suspected to be important in the mechanism involved in hormonal progression of CaP (2). The 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 the AR and alternative signal transduction pathways (3).

To explore the mechanism by which the bone microenvironment may promote the 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 cells, 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 from

fibroblasts (FCM) were prepared (3, 4). Briefly, bone chips from femoral head trabecular explants were cultured in MEM containing 20% FBS. Osteoblast-like cells outgrown were characterized using the reverse transcriptase-PCR for expression of the osteoblast markers type I procollagen, alkaline phosphatase and osteocalcin (5). OCM was collected after culturing of osteoblast-like cells for 48 hours in serum-free MEM. B26, B33 and B44 refer to OCM prepared from bone samples from patient 26, 33 and 44 respectively.

Cell Proliferation Analysis

LNCaP, 22RV1 and RKO cells in 96-well Falcon Primaria tissue culture plates (60% confluence) treated with: 0.00000385% v/v Ethanol (vehicle control), 0.1nM R1881, OCM (50% v/v) or FCM (50% v/v). After 5 days, cell proliferation was assessed using a BrdU ELISA kit (Roche Diagnostics, Laval, Canada).

Flow Cytometry

LNCaP cells were treated with ethanol, 0.1nM R1881 or OCM (50% v/v) in serum-free and phenol red-free RPMI 1640 (sfRPMI 1640). 48 hours later, cells were treated with 100 μ l of 1 μ g/ml of BrdU for 1h at 37°C. BrdU staining was performed (BD Biosciences, San Jose, CA, USA) and cells were analyzed by FACS Calibur (BD Biosciences) and Flow-Jo software (Tree Star, Inc., Ashland, USA).

Fluorescent Microscopy

LNCaP cells (5×10^4) treated with EtOH, 0.1nM R1881, or OCM (50% v/v) in sfRPMI 1640. After 3 days, cells were stained using the ApopTag Fluorescein apoptosis detection kit (Chemicon International, Hampshire, U.K.), counterstained with DAPI, and examined using an Axioplan-2 Fluorescence Microscope (Zeiss, Toronto, Canada).

Animal Models

The LNCaP hollow fiber model was performed (6, 7) with modifications. Briefly, hollow fibers containing LNCaP cells solely or co-cultured with osteoblasts (1×10^6 each) were subcutaneously 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, Montreal, Canada).

Affymetrix GeneChip Analysis

Cells were serum starved for 48 hrs and were 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, Santa Clara, CA). Hybridizations were completed independently for each condition using three biological replicates.

Expression Profile Analysis

Comparative analysis between expression profiles for Affymetrix experiments employed GeneSpring™ software version 7.2 (Silicon Genetics, CA). The expression profiles from 3

independent experiments were compared using Student two-sample t-test (parametric test, assume variances equal) to identify genes that were differentially expressed. Affymetrix datasets 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, PC 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 co-author RLV. Real time RT-PCR used gene-specific primers (SI Table 1) and SYBR Green Supermix (Invitrogen) in an ABI 7900 machine (Applied Biosystems, Foster City, USA) with cycling conditions: 95°C for 2 min; followed by 40 cycles of (95°C for 15 sec, 55°C for 30 sec). GAPDH-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 sRPMI for 24hrs after which cells were treated as in figure legends. Immunoblot analysis used cells lysed in SDS-PAGE sample buffer. STAT3 immunoprecipitation used anti-total STAT3 (Cell Signal). Blots were probed with antibodies against phosphor or total STAT3, ERK1/2 (Cell Signal), ErbB2 or AKT (R&D).

Results

Osteoblast-derived factors promote survival and proliferation CaP cells

To investigate the mechanism(s) underlying progression of CaP in response to osteoblast-derived factors in the absence of androgen and serum, we employed osteoblast-conditioned medium (OCM) and examined changes in proliferation of LNCaP and 22RV1 cells by BrdU incorporation. Osteoblast-like cells from different bone donors (e.g., B45, B66, B70) were used to obtain OCM. The proliferation of both CaP cell lines increased in the absence of androgen when treated with OCM (50% final concentration) as compared to the untreated control (Fig. 1a). Androgen (0.1nM R1881) increased the proliferation of both CaP cell lines as expected (positive control) (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 two-fold increase (~42%, $p<0.001$) in the percentage of cells in S-Phase as compared to the control group that had approximately 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 to the control. These data indicate that OCM increased the proliferation of CaP cells in the absence of androgen.

To evaluate the role 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, approximately 10% of the total population of LNCaP cells had positive staining for TUNEL (Fig. 1c). Androgen decreased staining for TUNEL to about 4% ($p<0.001$), while OCM-treated cells showed even further decrease in TUNEL staining to approximately 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 into mice subcutaneously (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 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 to 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 subcutaneous xenograft model (4) (SI Fig. 1).

Together these results: 1) mimick the clinical scenario whereby patients with bone lesions tend to have a higher PSA nadir than those patients with soft tissue involvement; and 2) 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 48h before total RNA was extracted and analysed using Affymetrix Genechips to determine the global gene expression signature. Significance analysis of microarrays (12) was applied to identify genes differentially expressed across the various conditions. Using a two-way analysis of variance ($P \leq 0.05$) and 1.5-fold change cut off, 181 genes were differentially expressed across the various conditions (SI Table 2). Of these genes, there were 40 with known function in cancer, apoptosis, and the cell cycle (SI Fig 2 and SI Table

3). 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 while 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 upregulation of apoptosis inhibitor MALT1, ESPL1, PAR1 and downregulation of apoptosis mediators F2R, BCAP29, CRADD and STK3. Consistent with increased proliferation in response to OCM, a number of cell cycle related genes that were differentially expressed in response to OCM. The genes known to function in particular phases of the cell cycle or checkpoints are shown (SI Fig 2d). 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 VEGF and cell cycle genes GTSE1, HCAP-G, AURKB, BUB1, BIB1B, MCM5, PLK1, CDKN3, ASNS, CHEK1, DEPDC1, ESPL1, SMC4L1 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.

Pro-survival and proliferative signaling pathways are activated in LNCaP cells that are exposed to osteoblast-derived factors

The 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 IL-6 and VEGF were consistently higher in OCM preparations compared with the levels measured in controls (SI Fig. 3). 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, while the expression of CCNB2, AURKB and CDC2 was blocked in two out of three OCM preparations (SI Table 4). 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 JAK/STAT3, MAPK, PI3K and ErbB2 pathways, all of which have been implicated in hormonal progression of CaP (2) (SI Fig. 4 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 LCM (Fig. 3aI/II). JAK2 activation may be upstream to these effects since its inhibitor, AG490, blocked

STAT3 phosphorylation (Fig 3aIII). Pre-incubation with IL-6 neutralizing antibody, but not with the isotype IgG control, blocked OCM-inducible STAT3 phosphorylation (Fig 3aIV). Consistent with STAT3 involvement, known gene targets of STAT3 including PIM1, BCL-2, VEGF and BIRC5/Survivin were upregulated at the mRNA level in OCM-treated LNCaP cells (SI Table 2; data not shown). OCM increases the PSA promoter reporter activity (3) and this was significantly decreased upon pre-treatment with non-toxic 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 3c). IL-6 neutralization did not completely block ERK phosphorylation nor was there any detectable effect on the activation of ErbB2 (SI Fig. 5a, b). OCM-inducible ERK activation was blocked by the ErbB2 inhibitor, AG879 and, as expected, by the MEK 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, while 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 the AR (3). Thus in the absence of androgen, osteoblasts may release factors that activate the 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 1092 genes previously identified to be differentially expressed in LNCaP cells treated with R1881 (7) and OCM (represented on each column). Comparison of expression profiles demonstrated 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, while 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 (SI Table 5). 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) while levels of expression of OSR2, PIK3R1 and 215441_at decreased by both androgen and OCM. Only expression of APPBP2, BRE and SOCS2 were regulated in opposite directions. These data support that a subset of androgen-regulated genes become re-expressed 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 that included benign (n=6), localized (n=7), and metastasis (n=6) (21) using a hierarchical 2-dimensional clustering algorithm based on similarity of expression patterns. Comparison of expression profiles demonstrated a strong tendency to subgroup the samples with respect to tumor stage (Fig 5a) and the metastases samples were more distinct from other stages. Consistent with our hypothesis, LNCaP cells treated with OCM clustered with the clinical metastases 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 5bI). 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 while 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 5bII). To confirm the value of this signature, we applied a bigger sample pool of 18 benign prostate tissues, 63 sub-benign samples from the tissues beside the primary tumor, 61 primary CaP as well as 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 5cI). For the class prediction analysis, most (3 of 25) of the samples of CaP metastasis had a positive OCM margin while most of the localized CaP sample (50 of 61), the sub-benign samples (61 of 63) and the benign prostate tissues (16 of 18) had a negative OCM margin (Fig 5cII). Our gene expression signature could resolve metastatic CaP from localized CaP with 89% accuracy ($p=1.66E-13$) as well as localized CaP from sub-benign tissues ($p=0.017$). Our gene expression signature could not distinguish the metastases samples from the primary samples of hypopharyngeal (GDS1070) and oral cancers (GDS1062) (data not shown), but did have prediction accuracy ~84% for melanoma metastasis (GSE8401) (SI Fig 6).

To aid in revealing how factors derived from osteoblasts may promote the metastatic phenotype in CaP, we more closely 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 upregulated in samples of metastases (SI Fig 7a). Apoptosis mediators, CRADD and BCAP29, were downregulated while apoptosis inhibitor ESPL1 was upregulated. All of the OCM-regulated cyclins (CCNA2, CCNB1, CCNB2 and CCNE2) and most of the OCM-regulated CDCs (CDC2, CDC6, CDC20 and CDC25C) were upregulated in metastases. Negative cell cycle regulator, ACPP, was downregulated while VEGF and cell cycle mediators (ASNS, AURKB, BUB1, BUB1B, CDKN3, CHEK1, DEPDC1, ESPL1, GTSE1, HCAP-G, MCM5, PLK1, SMC4L1, STK6) were upregulated 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 site of metastasis, we compared clinical castration-recurrent samples of bone metastasis ($n=6$) to lymph node metastasis ($n=6$). Comparison of expression profiles demonstrated 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 (Fig 1 & 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 (androgenin-dependent) ($n=10$) lesions (24). Upon comparison of expression profiles, samples appeared 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% (7/10) of the castration-recurrent samples have a positive OCM margin while 90% (9/10) of the androgen-dependent samples 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, SMC4L1 and STK3 (upregulated in castration-recurrent samples) and a candidate tumor suppressor gene ACPP (downregulated in castration-recurrent samples) (SI Fig 7b).

Discussion

CaP frequently metastasizes to the bone which is associated with hormonal progression (25). Here, the impact of exposure of CaP cells to osteoblast-derived factors yielded the following observations: 1) OCM promotes the survival and proliferation of CaP cells; 2) *in vivo*, osteoblasts promote hormonal progression of CaP; 3) the gene expression profile of CaP cells in response to OCM was identified; 4) OCM induced the transcriptional program in

CaP cells that partially overlapped with the transcriptional program for androgen; 5) a gene expression signature induced by OCM could distinguish between clinical metastasis of CaP and other stages of the disease; and 6) 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 induce androgen-independent proliferation of CaP cells maintained in cell culture and *in vivo* in castrated hosts (3, 8, 4, 9). In agreement with colon cancer 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 (SI Fig 8a/b) and large sample pools (SI Fig 8c/d), but did not perform as well as the gene expression signature based upon the 40 genes (the accuracy of 95% vs. 100% in smaller sample pool and 83% vs 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 upregulated, while ACPP, which mediates vitamin D induced cell growth arrest (28), was downregulated 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.

The 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 in response to OCM supports activation of AR pathway in the absence of androgens. 19 genes were differentially expressed in response to both androgen and in the absence of androgen by OCM (genes are summarized in SI Table 5). This number was higher than expected by chance ($181 \times 1092 / 54000 = 3.66$; 181 and 1092 are the numbers of OCM or androgen responsive genes respectively; 54000 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. The AR may be an important quality of CaP that facilitates clonal 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, $p=1.21714E-05$) 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 ($p=0.32$, SI Fig 9) 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 metastases samples while only 8 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 since 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 tumor 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. While 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Osteoblast-derived factors promote survival and proliferation of CaP

(A) Cell proliferation was assessed by BrdU incorporation. Fold-induction was calculated relative to ethanol-treated samples. (B) Cell cycle analysis of differentially treated cells using BrdU incorporation and FACS analysis. (C) Analysis of apoptosis of differentially treated cells by TUNEL staining (green) counterstained with propidium iodide (red). The bars represent the mean $\pm$ standard deviation. Student t-test: *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$.

Figure 2. Osteoblasts promote *in vivo* hormonal progression of CaP

H&E staining of LNCaP cells co-cultured with osteoblast-like cells (OB) (A) or without OB (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+OB (n=12) before and after castration (Cx). The bars represent the mean $\pm$ standard error. Student t-test: *, p < 0.05.

Fig 3**Figure 3. OCM-inducible signaling pathways**

(A) *I/II*, Levels of tyrosine phosphor (pTyr Stat3), serine phosphor (pSer Stat3) and total (tStat3) STAT3 in LNCaP cells treated with 50% of OCM, 50ng/ml IL-6 (positive control), and negative controls FCM and conditioned media from LNCaP (LCM). *III/IV*, cells were pretreated for 30 min with the JAK2 inhibitor, AG490 (50uM). IL-6 was blocked by pre-treating the OCM with IL-6 neutralizing antibody (anti-IL6, 10ug/ml). IgG served as an isotype control. (B) Phosphor (pErk1/2) and total (tErk1/2) ERK1/2 levels. (C) *I*, Phosphor ErbB2 (pErbB2) and phosphor AKT (pAKT) levels. *II*, cells were pretreated for 30min with the ErbB2 inhibitor, AG879 (20uM). CRP, a cross-reactive protein (loading control). (D) Effects of kinase inhibitors on OCM-induced ERK1/2 phosphorylation. Cells were pre-treated with inhibitors or vehicle (DMSO) for 30 minutes prior to treatment with 50% OCM.

Figure 4. Osteoblast-derived factors stimulate the androgen axis in CaP

Hierarchical clustering based on the androgen-responsive expression signature of all 6 samples treated with synthetic androgen (R1881) and 4 samples treated with OCM. Red color indicates high expression and a green color indicates low expression of a gene. The genes are represented by each row and the experimental samples are represented by each column.

Fig 5

Figure 5. OCM gene expression signature can distinguish various stages of CaP

Affymetrix datasets of clinical CaP were downloaded from PUBMED GEO (GDS1439 and GSE6919). Total of 40 OCM regulated genes from SI Table 2 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)** *I*, Principle component analysis distinctly clustered the clinical samples from GDS1439 into three groups which closely correlate to the three different stages (benign prostate tissue, localized and metastatic CaP). *II*, 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 t-test was applied to calculate the p -value for the difference between groups. **(C)** *I*, Principle component analysis distinctly clustered the clinical samples from GSE6919. *II*, Class prediction analysis applying the gene expression signature provided each clinical sample from GSE6919 with an individual OCM margin which represents the likelihood of exposure to OCM. Student 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. BM: bone metastasis; LM: lymph node metastasis.

Figure 6. OCM gene expression signature identifies castration-recurrent disease
Affymetrix datasets of clinical CaP were downloaded from PUBMED GEO (GDS1390). Total of 40 OCM regulated genes from SI Table 2 were applied as the gene signature. (A) Sample clustering to measure the similarity of the expression pattern between different samples according to the gene expression signature induced by OCM. (B) Principle component analysis clustered the clinical samples into two groups which closely correlate to the two different stages (castration-recurrent/androgen-independent vs androgen-dependent). (C) Class prediction analysis applying the gene expression signature provided each clinical

sample with an individual OCM margin that represents the likelihood of exposure to OCM. Student t-test was applied.

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