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ALCAM/CD166 Is a TGF-β-Responsive Marker and Functional Regulator of Prostate Cancer Metastasis to Bone

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Abstract

The dissemination of prostate cancer to bone is a common, incurable aspect of advanced disease. Prevention and treatment of this terminal phase of prostate cancer requires improved molecular understanding of the process as well as markers indicative of molecular progression. Through biochemical analyses and loss-of-function in vivo studies, we demonstrate that the cell adhesion molecule, activated leukocyte cell adhesion molecule (ALCAM), is actively shed from metastatic prostate cancer cells by the sheddase ADAM17 in response to TGF-β. Not only is this posttranslational modification of ALCAM a marker of prostate cancer progression, the molecule is also required for effective metastasis to bone. Biochemical analysis of prostate cancer cell lines reveals that ALCAM expression and shedding is elevated in response to TGF-β signaling. Both in vitro and in vivo shedding is mediated by ADAM17. Longitudinal analysis of circulating ALCAM in tumor-bearing mice revealed that shedding of tumor, but not host-derived ALCAM is elevated during growth of the cancer. Gene-specific knockdown of ALCAM in bone-metastatic PC3 cells greatly diminished both skeletal dissemination and tumor growth in bone. The reduced growth of ALCAM knockdown cells corresponded to an increase in apoptosis (caspase-3) and decreased proliferation (Ki67). Together, these data demonstrate that the ALCAM is both a functional regulator as well as marker of prostate cancer progression. Cancer Res; 74(5): 1404-15. ©2014 AACR.

Introduction

Morbidity and mortality among patients with prostate cancer are frequently a result of metastatic dissemination to bone. To date, there is no curative treatment of skeletal metastasis and survival from the time of diagnosis is merely 3 to 5 years (1). Moreover, skeletal events are associated with high morbidity in the form of bone loss, fractures, and pain (2). Even for patients with organ-confined disease, the risk of disease progression drives the rigor of clinical intervention. Patients have been shown to do well on a regimen of active surveillance, which avoids the morbidity associated with surgery. However, without a better understanding of the mechanisms that drive progression and biomarkers to predict the risk of progression, many patients and physicians are not comfortable pursuing this option, and instead opt for more invasive "definitive" interventions. Thus, clinical intervention would greatly benefit from further understanding of the molecular mechanisms that drive skeletal metastasis as well as molecular indicators that identify patients at risk of disease progression. Because cell motility is an important contributor to metastasis, the activation state of migratory mechanisms could be suitable for both therapeutic intervention as well as a biomarker of metastatic behavior.

Tumor cell metastasis to distant sites, including bone, is a multistep process. Cancer cells must first detach from the primary tumor site and migrate locally to invade blood vessels. Thereafter, tumor cells intravasate into the bloodstream and are attracted to preferred sites of metastasis through site-specific cellular and microenvironmental interactions (3, 4). Activated leukocyte cell adhesion molecule (ALCAM) is a cell adhesion molecule that engages in homotypic and heterotypic cell adhesion in a calcium-independent manner (5). It has been implicated in a number of adhesive and migratory behaviors including axonal guidance, leukocyte homing, and cancer metastasis. ALCAM can be proteolytically cleaved at the cell surface by ADAM17 causing the ectodomain to be shed. This shedding can be induced by ionomycin, phorbol 12-myristate 13-acetate (PMA), and EGFs (6). Proteolytic

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cleavage/shedding of cell surface proteins is a common regulatory mechanism that can alter the function and localization of transmembrane proteins such as cell adhesion molecules, growth factors, and growth factor receptors. This regulation can weaken cell adhesion and destabilize adhesions junctions as in the case of E-cadherin, through the loss of homotypic cell adhesion molecular interactions (7). The soluble ectodomain can act as a soluble antagonist, thereby competing for membrane-bound receptors or cell adhesion molecules. Although these processes are important in development, they have also been associated with tumorigenesis. Functionally, expression of the truncated, transmembrane fragment of ALCAM in BLM melanoma cells results in increased lung metastasis in vivo, whereas overexpression of a soluble extracellular ligand-binding fragment diminished metastases (for review see refs. 8 and 9). Previous studies from our laboratory and others have investigated the clinical relevance of ALCAM expression and shedding in a variety of human malignancies, including colorectal, breast, ovarian, thyroid, and prostate cancer (10–16). However, its molecular contribution to disease progression remains unclear.

Elevated ALCAM expression in aggressive prostate cancer (17) together with its putative role in cell adhesion/migration (8, 18) suggested that ALCAM is a molecular participant in prostate cancer metastasis to bone. On the basis of our own work in colorectal cancer (10) and concomitant work in breast (19) as well as ovarian cancer (11), we hypothesized that ectodomain shedding of ALCAM is likely to correspond with prostate cancer progression. Furthermore, considering the dominant role of TGF-β in driving skeletal metastasis of prostate cancer (20, 21), we postulated that this cytokine could influence ALCAM expression and/or shedding. The present study investigates ALCAM expression and shedding during prostate cancer progression, evaluates the influence of TGF-β stimulation, and determines the contribution of ALCAM to skeletal metastasis in a series of orthotopic, and experimental metastasis models.

Materials and Methods

Reagents, cell culture

Full-length purified recombinant porcine TGF-β was obtained from R&D Systems. Antibodies against ALCAM were obtained from R&D Systems (Clone 105902). The following tumor cell lines were obtained from American Type Culture Collection (ATCC) and maintained according to the ATCC’s recommendations: DU145, LNCaP, and PC3 (prostate metastasis), PC3-luciferase (PC3-luc) cells (Simon Hayward, Vanderbilt University, Nashville, TN) were cultured in RPMI/10% FBS.

SDS-PAGE and immunoblotting

Cells (2.5 × 10⁵) were plated in 6-well plates. After 24 hours, cells were serum-starved in Opti-MEM for 16 hours and then treated in the presence or absence of indicated growth factors or inhibitors for 48 hours. After that, the cells were lysed in TNE lysis buffer [20 mmol/L Tris–Cl (pH 7.4), 0.5 mmol/L EDTA, 1% Triton X-100, 150 mmol/L NaCl, protease inhibitor cocktail (Sigma)]. 1 mmol/L phenylmethylsulfon fluoride]. Total protein in the lysates was quantified by using the bicinchoninic acid (BCA) assay (Bio-Rad). Conditioned media samples were concentrated with Microcon centrifugal filters (Millipore) following the manufacturer’s protocol, which were eluted directly in 5× sample buffer for Western blot analysis. Protein loading for conditioned medium samples for Western blot analysis was adjusted according to the total protein in cell lysates. Conditioned media and total protein was subjected to SDS-PAGE and electrophoretic transfer to polyvinylidene difluoride membranes (Immobilon P, Millipore, Inc.). Immunodetection was done by conventional chemiluminescence.

Quantitative PCR

The mRNA samples were prepared from tumor cells lysed in TRI Reagent (Ambion) and purified using phenol extraction, followed by real-time PCR (RT-PCR). The following quantitative PCR (qPCR) primers were used ALCAM, TCAAGGTGTTCGAACCA (forward) and CTGAAATGACGTACCCAC (reverse); ADAM17, ATGTCTACGGTGGCGTCA (forward) and CATGTATCGTGAGCAATGC (reverse); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ATCTTCTTTGGTGGCCG (forward) and TTCCCCATGGTGTCGAC (reverse).

shRNA knockdown

To establish cell lines in which ALCAM expression is stably knocked down, cells were transduced with ALCAM-specific Mission short hairpin RNA (shRNA; Sigma) lentivirus. Following transduction, cells were selected in 10 μg/mL of puromycin. Transduced cells were flow sorted for ALCAM expression and ALCAM knockdown cells were cultured and maintained in 5 μg/mL of puromycin.

Migration assay

Two-dimensional gap closure assays (formerly known as scratch assays) were conducted by using magnetically attachable stencils attached to culture plates (22). In short, 250,000 cells were seeded in 6-well plates and allowed to recover overnight to form a confluent monolayer. Stencils were removed with tweezers, after which cells were rinsed with PBS to remove detached cells. Culture medium was re-added and closure of the gap was measured at 8 and 16 hours. Gap closure was quantified using TScratch (NIH, Bethesda, MD).

Histologic analysis of mouse tissue

Tumor-bearing tissue and bones were fixed in 10% formalin. Bone specimens were decalcified in 20% EDTA pH 7.4 for 3 to 4 days at room temperature. Decalcified bone and tissue were dehydrated and embedded in paraffin. Tumor burden was confirmed in 5-μm serial sections stained with hematoxylin and eosin (H&E). Osteoclasts were visualized using a standard tartrate-resistant acid phosphatase (TRAP) protocol. All immunohistochemistry and immunofluorescence on tumor sections involved antigen retrieval by using a standard pH 6.0 citrate buffer followed by blocking via incubation with 20% Aquablock (East Coast Bio). Immunofluorescence data were obtained using primary antibodies for ALCAM (1:1,000; Leica Biosystems; Clone, MOG/07), Ki67 (1:50; Fisher; Clone SP6), cleaved caspase-3 (1:200; Cell Signaling Technology; D175), and
collagen I (1:1,000; Sigma; C2206) by incubation overnight at 4°C. Corresponding Alexa Fluor secondary antibodies were used (1:1,000; Invitrogen). Fluorescent imaging was collected on an Olympus BX61WI upright fluorescent microscope by using Volocity Imaging Software.

**ELISA of mouse serum and plasma**

Blood was obtained via the saphenous vein; samples were collected in either the presence of EDTA as an anticoagulant or a serum separator tube (Fisher Scientific; cat#1491559 and 1491553, respectively) and were centrifuged at 1,500 rpm and 4°C to remove cells. Plasma and serum samples were stored at −80°C until analyzed. Samples were analyzed for soluble mouse and human ALCAM using the R&D Systems DuoSet following the manufacturer’s instructions. Briefly, ELISA plates coated with capture antibody were incubated overnight with 100 μL of sera diluted at 1:50. Capture ALCAM was detected with biotinylated antibody and peroxidase-conjugated avidin followed by colorimetric detection at 450 nm.

**Mouse models of prostate cancer and in vivo quantitation of tumor growth**

All experimental protocols were approved by the Vanderbilt University Institutional Animal Care and Use Committee. Orthotopic prostate xenografts were performed according to Li and colleagues (23). Briefly, 5 × 10^4 PC3-luc cells were suspended in 30 μL of neutralized type I collagen and allowed to polymerize for 16 hours at 37°C before implantation into the prostate of 10-week-old C57/129ScidPrkdcsevere combined immunodeficient mice (SCID) male mice (Harlan Laboratories). Tumor growth was monitored weekly by bioluminescent detection of luciferase-expressing cells. For the xenograft model, subconfluent PC3-luc cells were trypsinized, washed twice in PBS to remove serum, and then resuspended in Hank’s Balanced Salt Solution (GIBCO) at a concentration of 1 × 10^5 cells/mL. Of note, 100 μL containing 1 × 10^6 PC3 cells in a 50/50 mix of PBS and growth factor–reduced Matrigel (BD Biosciences) were injected subcutaneously into the right flank of 7-week-old nude male mice (Harlan Laboratories; athymic Foxn1 nu/nu). Tumor growth was monitored weekly by caliper measurements, and tumor volume was calculated on the basis of the following formula: (length × length × width)/6. PC3-luciferase shControl (Vector) or PC3-luciferase shALCAM (KD2 or KD3) tumor cells (1 × 10^5) in a 10 μL volume of sterile PBS were injected into the tibia of anesthetized 6-week-old nude male mice (Harlan Laboratories). Skeletal metastasis was performed as previously described by Park and colleagues (24) and as visualized in ref. 25. Briefly, 1 × 10^6 PC3-luc cells were injected into the left heart ventricle of male nude mice (Harlan Laboratories). Skeletal metastases were monitored by bioluminescent detection of luciferase-expressing cells and formation of bone lesion by X-ray. Whole-animal luminescent imaging was performed with the IVIS system (Caliper Life Sciences). Luciferin (150 mg/kg in sterile PBS; Biosynth International) was delivered via intraperitoneal injection 10 minutes before imaging. Living Image software (Caliper Life Sciences) was used to quantify the luminescence intensity. Blood was obtained via the saphenous vein and collected in either the presence of EDTA as an anticoagulant or a serum separator tube (Fisher Scientific). Plasma and serum samples were stored at −80°C until analyzed.

**Micro computed tomography analysis**

For gross analysis of trabecular bone volume, formalin-fixed tibiae were scanned at an isotropic voxel size of 12 μm by using a micro computed tomographym 40 (μCT40; SCANCO Medical). The tissue volume was derived from generating a contour around the metaphyseal trabecular bone that excluded the cortices. The area of measurement began at least 0.2 mm below the growth plate and was extended by 0.12 mm. The bone volume included all bone tissue that had a material density greater than 438.7 mg HA/cm^3.

**Radiographic analysis**

Beginning 1 week after tumor cell inoculation, tumor-bearing animals were subjected to radiographic imaging. Radiographic images (Faxitron X-ray Corp.) were obtained by using an energy beam of 35 kV and an exposure time of 8 seconds. Osteolytic lesions were quantified bilaterally in the Tibia, fibula, femora, humeri, and pelvis at the endpoint using X-ray images. Lesion area and lesion numbers were evaluated by using image analysis software (Metamorph: Molecular Devices, Inc.). Data presented are the average of lesion area and lesion numbers per mouse in each group.

**Statistical analyses**

Expression analysis was performed on datasets GDS1439 and GSE10645 available through the Gene Expression Omnibus (refs. 26 and 27, respectively). Expression data for selected genes from GDS1439 was clustered in software Cluster 3.0 and visualized with software TreeView. For survival analysis, the patient population of GSE10645 (n = 596) was dichotomized across upper and lower quartile of ALCAM expression. Statistics were completed by using either R, SPSS, or GraphPad Prism. For all standard bar and box plots, the results were reported as mean and SEM, unless stated otherwise in the legend. Comparisons were performed using unpaired two-sided Student t test, nonparametric Mann–Whitney test, or one-way ANOVA. R^2 and P values were reported from linear regression analysis of mouse data. All statistical tests were considered significant when P < 0.05; *, P < 0.05; **, P < 0.01; and ***, P < 0.001.

**Results**

ALCAM gene expression is elevated in advanced prostate cancer and correlates with poor patient outcome

Changes in ALCAM expression have been linked to patient outcome for several malignancies. In prostate cancer, the correlation of ALCAM expression with patient outcome is sometimes conflicting. Minner and colleagues (15) conclude that reduced ALCAM expression correlates to poor patient outcome, whereas the opposite was suggested by Kristiansen and colleagues (17). We evaluated several publicly available microarray datasets to determine the relationship between ALCAM mRNA levels, patient diagnosis, and outcome (Fig. 1). ALCAM expression seems to be elevated in an experimental
model of epithelial–mesenchymal transition performed by the Weinberg’s laboratory [(28) GSE9691; Supplementary Fig. S1]. Indeed, a comparison of benign, localized, and metastatic prostate cancer revealed that the level of ALCAM mRNA increased in metastatic disease (Fig. 1A; GDS1439) and coincided with molecular evidence of a promigratory phenotype based on the decreased expression of E-cadherin and p120 with concurrently elevated expression of N-cadherin (Fig. 1B). These observations were supported by survival analysis for a cohort of 596 patients with prostate cancer (GSE10645), which revealed that the high levels of ALCAM mRNA corresponded with poor patient outcome (Fig. 1C). Immunohistologic staining of prostate cancer tissue microarrays available through the Human Protein Atlas (29) revealed that ALCAM staining is clearly evident in both normal, low-, and medium-grade disease but is frequently absent from the tumor cell surface in high-grade disease (proteinatlas.org; Fig. 1D).

**TGF-β** induces **ALCAM** expression and shedding

Because ALCAM is associated with disease progression, we set out to determine its contribution to the skeletal metastasis of prostate cancer. Moreover, because bone metastasis is driven in large part by TGF-β (2, 20, 21), we investigated the ability of this cytokine to promote ALCAM shedding in vitro. ALCAM is proteolytically shed from PC3 cells (Supplementary Fig. S2). Absence of the cytoplasmic tail in the conditioned medium confirms that the ectodomain is shed (Supplementary Fig. S2A). Moreover, ALCAM is absent from PC3-derived exosomes, ensuring that the ectodomain is shed and not released with cell-derived microparticles (Supplementary Fig. S2B). Selected cytokines thought to be involved in metastasis to bone, including TGF-β, were tested for their ability to alter ALCAM shedding (Fig. 2A). ALCAM shed into the conditioned media is detected by ELISA and normalized to the amount of cellular ALCAM detected in the lysate. Of the eight agents tested, only TGF-β
was able to promote ALCAM shedding. To further explore the response to exogenous stimulation with TGF-β, ALCAM expression in PC3 cells was compared with ALCAM expression in LNCaP cells that are unable to respond to the cytokine because they lack TGF-β receptor type I (Fig. 2B and C; ref. 30). qRT-PCR analysis for ALCAM demonstrates that TGF-β was also able to induce ALCAM gene transcription in PC3 but not LNCaP cells (Fig. 2B). The absence of any significant increase in lysate ALCAM further supports cytokine-induced ALCAM shedding (Fig. 2C). Conversely, LNCaP did not respond to TGF-β even though these cells express abundant ALCAM (Fig. 2C). TGF-β–induced expression in PC3 cells could be abrogated with the small-molecule inhibitor SB431542 (Fig. 2D, 10 μmol/L; Sigma), whereas TGF-β–induced ALCAM mRNA and protein expression could be restored in LNCaP cells when the cells were transfected with dominant-active TGF-β receptor type I (Fig. 2E and F).

**ALCAM shedding in vivo correlates with tumor progression**

Published clinical studies have demonstrated that circulating levels of ALCAM are frequently elevated in patients with cancer (19, 31, 32). These studies suggest that ALCAM is shed by the tumor. Indeed, experimental models of ovarian cancers indicate elevated shedding of ALCAM specifically from the tumor (11). To determine whether tumor-derived ALCAM is the source of elevated circulating ALCAM in prostate cancer,
we used species-specific antibodies to monitor circulating levels of both host (mouse) ALCAM and tumor (human) ALCAM longitudinally during orthotopic and subcutaneous growth of PC3 cells (Fig. 3). To determine that tumor-derived ALCAM could act as a stable biomarker of cancer in vivo, we determined the half-life of human ALCAM in the circulation of its mouse host (Supplementary Fig. S3). Circulating ALCAM exhibits a 17-hour half-life that is sufficient for monitoring its release from an endogenous tumor burden.

Circulating levels of ALCAM were subsequently monitored on a weekly basis (Fig. 3A) in SCID mice bearing subcutaneous (Fig. 3B, n = 5) or orthotopic xenografts of PC3 (Fig. 3C, n = 8). Animals were bled on a predetermined schedule via saphenous vein puncture. Circulating ALCAM levels were detected by ELISA and a comparison with pregrafting baseline levels allowed for the detection of any increase in host (mouse) ALCAM and the appearance of tumor (human) ALCAM in response to an increasing tumor burden. Tumor-derived ALCAM levels showed significant weekly increases in the serum of tumor-bearing mice (Fig. 3B and C, and Supplementary Fig. S4A; P < 0.0001). Regression analysis showed a direct linear relationship between circulating levels of tumor-derived ALCAM and tumor burden for subcutaneous xenografts (Fig. 3B; tumor-derived $R^2 = 0.707; P < 0.0001; n = 4$) and orthotopic xenografts (Fig. 3C; tumor-derived $R^2 = 0.7066; P < 0.0001; n = 4$). In contrast to tumor-derived ALCAM, changes in host-derived ALCAM did not correspond to tumor burden (Fig. 3B; host-derived $R^2 = 0.03671, n = 4$ animals; Fig. 3C; host-derived $R^2 = 0.01358, n = 4$ animals).

A significantly greater amount of circulating ALCAM was observed in mice bearing PC3 versus mice bearing LNCaP tumors (Supplementary Fig. 5B), supporting the relationship between malignancy and ALCAM shedding seen in vitro (Fig. 2C). Moreover, shedding increased for tumor cells selected from skeletal metastases (Supplementary Fig. 5F). Shedding is likely to be universally present in solid tumors as it has been reported for colon cancer (10), ovarian cancer (11), and is easily detected in models of breast and prostate cancer (Supplementary Fig. S4C–S4E).

ALCAM shedding is mediated by ADAM17 in vitro and in vivo

Because ALCAM is a proteolytic target of ADAM17, we hypothesized that ADAM17 was responsible for TGF-β-induced cleavage of ALCAM. This hypothesis was supported by published work demonstrating that TGF-β can increase ADAM17 activity by phosphorylation of the protease (33, 34). Indeed, knockdown of ADAM17 using siRNA transfection resulted in a loss of TGF-β–induced ALCAM shedding (Fig. 4A). Similar results were obtained by using an ADAM17-specific inhibitor (Fig. 4B, Compound 32, BMS; ref. 35). These studies were
Similar observations were made in ALCAM KD2 and ALCAM KD3 TGF-β attachable stencils (Fig. 5B; ref. 22). The analysis revealed a loss of shedding is mediated by ADAM17 and promoted by TGF-β. Taken together, these data suggest that ALCAM cell surface shedding is achieved with our dosing studies (Supplementary Fig. S5). Serum levels of shed ALCAM, approximating the 50% inhibition of ADAM17 resulted in a significant decrease in inhibition of ADAM17 (Fig. 4C). In vivo dosing and efficacy for Compound 32 was confirmed by using serum TNF-α (Supplementary Fig. S5, n = 6), which demonstrated that 50% inhibition of ADAM17 could be achieved for the duration of the ALCAM serum half-life (Supplementary Fig. S3; 17 hours) without signs of distress or toxicity. Mice were treated twice daily for 3 days with 20 mg/kg of the ADAM17 inhibitor [Compound 32 or vehicle dimethyl sulfoxide (DMSO) control]. Presurgery, weekly, pre-, and post-treatment saphenous vein bleeds were collected. We found that inhibition of ADAM17 resulted in a significant decrease in serum levels of shed ALCAM, approximating the 50% inhibition we achieved with our dosing studies (Supplementary Fig. S5). Taken together, these data suggest that ALCAM cell surface shedding is mediated by ADAM17 and promoted by TGF-β.

**Knockdown of ALCAM in PC3 cells inhibits TGF-β-induced migration and in vivo dissemination to bone**

Given that TGF-β is a central driver of tumor cell motility and metastasis, we sought to determine the effects of exogenous TGF-β on prostate cancer cells in vitro. To test whether ALCAM is functionally involved in tumor cell migration and metastasis, we knocked down expression in PC3-Luc cells using viral delivery of shRNA. Three separate stable ALCAM knockdowns were produced (ALCAM KD 1, 2, and 3). Transduced cells were selected with puromycin and subsequently subjected to flow-sorting to isolate the highest knockdown population (Fig. 5A). We pretreated PC3-luc-ALCAM KD3 cells and PC3-luc-ALCAM shControl cells with 10 μg/mL TGF-β1 for 16 hours in serum-free conditions, followed by the analysis of migration by magnetically attachable stencils (Fig. 5B; ref. 22). The analysis revealed a loss of TGF-β–induced migration in PC3-luc-ALCAM KD3 cells (Fig. 5B). Similar observations were made in ALCAM KD2 and ALCAM KD3 PC3 cells as well as in MDA-MB-231 and A549 cells that represent breast and lung cancer, respectively (Supplementary Fig. S6). Interestingly, the reduction in ALCAM expression led to a slight but statistically insignificant increase in spontaneous migration, possibly due to a loss of ALCAM–ALCAM homotypic interaction on adjacent cells.

Knockdown of ALCAM in PC3 cells diminishes tumor growth in bone

Given the critical importance of cancer cell migration in malignant tumor expansion and metastasis, we subsequently evaluated the contribution of ALCAM to primary tumor growth and bone metastasis. Primary tumor growth was accomplished by using an orthotopic model based on implantation of tumor cells into the anterior prostate of SCID mice (Fig. 5C, n = 8 for PC3-luc-Control and PC3-luc-ALCAM KD1). Skeletal metastasis was accomplished by intracardiac injection of tumor cells in nude mice (Fig. 5D, n = 31 for PC3-luc-Control and n = 25 for PC3-luc-ALCAM KD1). Bioluminescent imaging was used to monitor tumor burden for both models at weekly intervals. Interestingly, reduced ALCAM expression did not limit tumor growth within the prostate (Fig. 5C). Whole body and ex vivo bioluminescent imaging of the orthotopic model upon experiment completion confirmed that both the PC3-luc-Control and PC3-luc-ALCAM KD1 exhibited similar tumor burden based on luciferase activity (Fig. 5C), and comparable tumor size based on weight (Fig. 5D). Local invasion and mesenteric dissemination is common in this model (36) and was not altered by reduced ALCAM expression.

In contrast to the orthotopic model, a reduction in ALCAM resulted in a significant decrease in skeletal metastasis (Fig. 5E). Both incidence and metastatic burden were reduced. Approximately, 75% of mice injected with PC3-luc-Control tumor cells developed bone metastasis, whereas only 17% of the mice injected with PC3-luc-ALCAM KD1 cells developed bone lesions. In addition, in mice that did develop skeletal metastases formed by PC3-luc-ALCAM KD1, the number of lesions per mouse was greatly reduced (0.2 events vs. 1.4 events; Fig. 5F). The orthotopic and intracardiac experiments were repeated with PC3-luc-ALCAM KD2 and similar results were obtained.

Knockdown of ALCAM in PC3 cells diminishes tumor growth in bone

To determine the biologic importance of tumor-derived ALCAM in prostate tumor growth in the bone, PC3-luc-Control
Mice from PC3-luc (n = 8 weeks after intracardiac injection of PC3-luc parental (shControl) or PC3-luc ALCAM knockdown (shALCAM, KD1). F, average number of bone lesions in mice bearing orthotopic PC3-luc parental tumors or PC3-luc ALCAM knockdown tumors 6 weeks after surgery. D, primary tumor weights of orthotopic PC3-luc parental tumors (n = 8) and PC3-luc ALCAM knockdown tumors (n = 8), E, representative whole-animal luciferase imaging and matching X-rays of mice bearing orthotopic PC3-luc parental tumors (shControl) or PC3-luc ALCAM knockdown (shALCAM, KD1).

**Discussion**

The data presented here reveal for the first time that ALCAM plays a major role in prostate cancer establishment in the bone microenvironment. ALCAM mRNA is elevated in malignant disease, yet by immunohistochemistry it is frequently absent from the tumor cell surface in advanced disease. Regression analysis showed a direct linear relationship between circulating levels of ALCAM and tumor burden in animals with subcutaneous xenografts of PC3-luc ALCAMKD3 tumors. The reduced tumor burden after intratibial injection suggested a diminished capacity to grow in the bone. Detailed imaging of the osteolytic lesions by μCT further confirmed decreased lesion area and increased bone volume in the bones containing ALCAM KD cells (Fig. 6B). Histologic visualization of bone tumors generated by control and ALCAM KD cells resulted in reduced bone tumor size upon ALCAM knockdown (Fig. 7D), however, both control and ALCAM KD tumors were osteolytic as evidenced by positive staining for the osteoclast marker, tartrate-resistant alkaline phosphatase (Supplementary Fig. S7).

To assess changes in survival and proliferation, bone tumors were stained for cleaved caspase-3 (Fig. 7E, apoptosis) and Ki67 (Fig. 7F, proliferation), respectively. Compared with the control tumors, the bone tumors created by both ALCAM KD cells exhibited elevated levels of cleaved caspase-3, suggesting that these cells are experiencing a reduced ability to survive (Fig. 7G). In addition, bone tumors from PC3-luc-ALCAMKD3 had significantly lower Ki67 staining, indicating that reduced ALCAM expression diminished the ability of PC3 cells to proliferate (Fig. 7H). These observations indicate that ALCAM contributes to both proliferation and survival.
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Figure 6. Tumor-derived ALCAM impacts metastatic growth but not incidence after intratibial injection. A, representative whole-animal luciferase and X-ray imaging of mice postintratibial injection of PC3-luc parental tumors (vector, n = 8), and PC3-luc ALCAM knockdown tumor cells (KD2, n = 8 and KD3, n = 8). B, bioluminescent curve of intratibial tumor development in mice bearing PC3-luc vector, PC3-luc KD2, and KD3 tumors (two-way ANOVA with Bonferroni posttest). C, tumor incidence and average lesion area in the tibias of mice bearing PC3-luc vector, PC3-luc KD2, and KD3 tumors. Data represent the mean ± SEM (n = 8/group); **, P < 0.01; *** , P < 0.0001. D, collagen I and ALCAM immunofluorescence of tumor cells within the tibias of mice bearing PC3-luc vector or PC3-luc KD2 or KD3 tumors.

(Fig. 3B) and orthotopic xenografts (Fig. 3C). In contrast, the host-derived ALCAM did not correspond to tumor burden (Fig. 3B and C), demonstrating that elevations in circulating ALCAM are tumor-specific. Consistent with this observation, host-derived ALCAM does not increase, but rather decreases slightly, in immunocompetent mice challenged with lipopolysaccharide (LPS, a model of acute inflammation) or full-thickness skin punch (a model for wound-healing Supplementary Fig. S8A and S8B, respectively). These data suggest that tumor-derived ALCAM is a marker specific of tumor burden and that host ALCAM is not significantly shed in response to the tumor burden.

Suppression of ALCAM expression using gene-specific shRNAs prevented TGF-β–induced migration in vitro (Fig. 5B) and inhibited metastasis as well as tumor growth in bone in vivo (Figs. 5–7). These observations demonstrate that ALCAM is not only a marker of cancer progression but also a significant regulator of tumor cell migration and metastasis to bone. Within the metastatic cascade, there are many sequential steps that can contribute to the overall success of any single metastatic lesion. Evaluation of the primary tumor within the prostate did not reveal any deficiency in growth or local invasion. Conversely, experimental metastasis by intracardiac injection resulted in nearly 10-fold reduction of skeletal metastasis (Fig. 5). This reduced metastatic incidence suggests that ALCAM is required for dissemination to the bone. Further examination of tumor growth after intratibial injection (Fig. 6) revealed a significant inhibition in tumor growth without affecting the tumor incidence. The reduced metastatic incidence from circulating tumor cells (intracardiac injection) together with the reduced growth of the metastatic burden in the bone (intratibial injection) without further impact on incidence indicates that ALCAM contributes to metastatic dissemination as well as growth in the metastatic sites. It remains to be determined whether these two biologic contributions are controlled by distinct molecular mechanisms.

Previous work has shown that both soluble ALCAM-Fc and blocking ALCAM antibodies are able to decrease in vitro transendothelial migration of THP1 monocytes (39). Although we could not test the role of ALCAM in extravasation directly, it is possible that the requirement for transendothelial migration extends to metastatic tumor cells. The reduced metastatic incidence after intracardiac injections indeed supports that hypothesis (Fig. 5). The lesions that did arise from circulating PC3-luc-ALCAMKD cells were smaller than those generated from PC3-luc-Control cells, suggesting an additional contribution from ALCAM to metastatic growth. Indeed, metastatic lesions generated after intratibial injection of PC3-luc-ALCAMKD cells did not significantly impact tumor incidence (Fig. 6C) but did dramatically reduce expansion of the metastatic tumor burden (Fig. 7F).

Intratibial tumors generated by PC3-luc-ALCAMKD did not reexpress ALCAM (Fig. 5D), allowing us to further investigate any molecular disparities between metastatic lesions that expressed ALCAM and those that did not. The abundant presence of osteoclast activity by TRAP staining (Supplementary Fig. S7) in metastatic lesions of PC3-luc-ALCAMKD suggests that there is no deficiency in osteolysis. Nevertheless, these lesions remain significantly smaller than those created by PC3-luc-Control, suggesting that there was not a lag in tumor growth but rather a persistent reduced ability to proliferate or, conversely, a decreased ability to survive. A review of the literature reveals one study that suggested that the loss of ALCAM may be associated with a proapoptotic behavior in...
breast cancer cells (40). Although we did not observe reduced proliferation in vitro (Supplementary Fig. S9), in vivo metastatic lesions created by PC3-luc-ALCAMKD2 did exhibit reduced proliferation and increased apoptosis. Intriguingly, PC3-luc-ALCAMKD2, which retains more ALCAM expression than PC3-luc-ALCAMKD3 (Fig. 6A), did not exhibit reduced proliferation, suggesting that the threshold of ALCAM expression that influences cell survival is different from the threshold in influencing cell proliferation.

Together, these observations suggest a dual adhesive and signaling function of ALCAM, whereby the intracellular signaling function of ALCAM functions to serve a protective role in apoptosis, and the extracellular adhesive function is required for extravasation and subsequent colonization at a secondary site. Although we have convincing data that ALCAM expression is important in the osteotropism and survival of tumor cells in the bone (Figs. 5 and 7, respectively), further work is necessary to fully elucidate the mechanisms involved.

Conclusion

Here, we have presented evidence that ALCAM not only serves as a longitudinal marker of tumor burden, but also contributes functionally to skeletal metastasis. Clinically, this may be of particular importance for prostate cancer in which metastasis to bone is a frequent aspect of end-stage disease. Monitoring ALCAM status may provide an indicator of metastatic potential and the ability to monitor metastatic burden. The relevance of ALCAM in the bone microenvironment is coupled with our data showing that ALCAM expression and
sheding is driven by TGF-β, a known contributor of the vicious cycle in bone metastasis (2, 41, 42). Further elucidation of the mechanism by which ALCAM supports metastatic dissemination to bone can provide novel means of promoting cytotoxic therapy in patients with skeletal metastases.

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No potential conflicts of interest were disclosed.

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