Cancer Stem-like Cell Marker CD44 Promotes Bone Metastases by Enhancing Tumorigenicity, Cell Motility and Hyaluronan Production

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Abstract

CD44, an adhesion molecule that binds to the extracellular matrix, primarily to hyaluronan (HA), has been implicated in cancer cell migration, invasion, and metastasis. CD44 has also recently been recognized as a marker for stem cells of several types of cancer. However, the roles of CD44 in the development of bone metastasis are unclear. Here, we addressed this issue by using bone metastatic cancer cell lines, in which CD44 was stably knocked down. Tumor sphere formation and cell migration and invasion were significantly inhibited by CD44 knockdown. Furthermore, the downregulation of CD44 markedly suppressed tumorigenicity and bone metastases in nude mice. Of note, the number of osteoclasts decreased in the bone metastases. Microarray analysis revealed that the expression of HA synthase 2 was downregulated in CD44-knockdown cells. The localization of HA in the bone metastatic tumors was also markedly reduced. We then examined the roles of CD44–HA interaction in bone metastasis using 4-methylumbelliferone (4-MU), an inhibitor of HA synthesis. 4-MU decreased tumor sphere and osteoclast-like cell formation in vitro. Moreover, 4-MU inhibited bone metastases in vivo with reduced number of osteoclasts. These results collectively suggest that CD44 expression in cancer cells promotes bone metastases by enhancing tumorigenicity, cell migration and invasion, and HA production. Our results also suggest the possible involvement of CD44-expressing cancer stem cells in the development of bone metastases through interaction with HA. CD44–HA interaction could be a potential target for therapeutic intervention for bone metastases.
Introduction

CD44, a single-chain, single-pass, transmembrane glycoprotein, is a major adhesion molecule for the extracellular matrix that binds primarily to the extracellular glycosaminoglycan hyaluronan (HA) (1, 2). CD44 has been implicated in a wide variety of physiological processes, including leukocyte homing and activation, wound healing, and cell migration as well as tumor cell migration, invasion, and metastasis. CD44 has also been recently recognized as one of the cell surface markers associated with cancer stem cells (CSCs), also referred to as cancer-initiating cells, in several types of cancers (2, 3). Although the nature of CSCs is still controversial, CD44-expressing subfractions of many human carcinomas are highly malignant and share common properties with CSCs. CD44-positive CSC-like cells have been shown to possess enhanced ability for metastasis (4, 5).

Mandal et al. showed that the inhibitory effects of simvastain and fish oil on bone metastasis are associated with the reduced expression of CD44 (6, 7). Furthermore, clinical studies have suggested a positive correlation between CD44 expression and bone metastasis (8, 9). These findings suggest that CD44 has roles in the development of bone metastasis; however, the underlying mechanisms are unclear.

HA, a ubiquitous extracellular and cell surface-associated matrix, is a high-molecular-weight linear glycosaminoglycan composed of repeating disaccharides of glucuronic acid and N-acetylglucosamine (10). HA synthesis and degradation are regulated by the enzymes HA syntheses (HASs) and hyaluronidases, respectively (10). Three isoforms of HAS, HAS1, HAS2, and HAS3, and 6 associated genes for hyaluronidases have been identified thus far. HA concentrations are usually elevated in malignant tumors compared with corresponding benign or normal tissues and are negatively correlated with clinical outcomes (11, 12). HA has also been shown to promote proliferation, invasion, and metastasis of cancer cells (10-12).
In this study, we examined the roles of CD44 in the development of bone metastases in a well-characterized animal model by establishing CD44-knockdown cancer cells. The results showed that CD44 expression in cancer cells promotes bone metastasis by enhancing tumorigenicity, and cell migration and invasion, and also through an HA-dependent mechanism. This study provides an initial glimpse of the functional meaning of a cell surface protein that has been widely associated with stem-like cell properties in human cancer.
Materials and methods

Reagents

Rat monoclonal antibody against CD44 (phycoerythrin-conjugated), mouse monoclonal antibody against CD24 (fluorescein isothiocyanate-conjugated), and isotype-matched IgG control were purchased from eBiosciences, Inc. (San Diego, CA). The rabbit polyclonal antibody against CD44 has been described previously (13). Biotinylated HA-binding protein (HABP) was purchased from Hokudo Co., Ltd. (Hokkaido, Japan). 4-Methylumbelliferone (4-MU) was purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals used in this study were purchased from Sigma-Aldrich or Wako Pure Chemical Industries, Ltd. (Osaka, Japan), unless otherwise specified.

Cell cultures

The human breast cancer cell lines MDA-MB-231 and MCF-7 and the human melanoma cell line A375 were obtained from the American Type Culture Collection (Rockville, MD). The human prostate cancer cell line PC-3 and the human lung cancer cell line HARA-B were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Miyagi, Japan) and the Health Science Research Resources Bank (Osaka, Japan), respectively. Cell lines were authenticated by the cell banks with short tandem repeat analysis. The cells were expanded and stored according to the supplier’s instructions and used within 2 months of recovery of frozen aliquots. MDA-MB-231, MCF-7, and A375 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies Japan Ltd., Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; Life Technologies Japan Ltd.) and 100 μg/ml kanamycin sulfate (Meiji Seika Kaisha, Ltd., Tokyo, Japan). PC-3 and HARA-B cells were cultured in RPMI1640 (Life Technologies Japan Ltd.) supplemented with 10% FBS and 100 μg/ml kanamycin sulfate. The cells were maintained in a humidified atmosphere of 5% CO₂ in air.
Flow cytometry

The cells were stained with fluorescence-labeled antibodies and analyzed using a flow cytometer (Cytomics FC500; Beckman Coulter, Inc., Fullerton, CA). Cell surface-associated HA was determined as described previously (14).

CD44 knockdown

Oligonucleotides encoding short hairpin RNAs (shRNAs) that target standard exons in CD44 mRNA (shCD44-2 and shCD44-3) (15) were cloned into the plasmid vector pBAsi-hU6 Pur (Takara Bio Inc., Shiga, Japan). The plasmids were transfected into cells using the Nucleofector II Device (Lonza Japan Ltd., Tokyo, Japan), and the cells were cultured for 2 weeks in the presence of puromycin (0.25 μg/ml; Sigma-Aldrich). CD44<sup>low</sup> cells were then sorted using a fluorescent-activated cell sorter (FACS; FACSAria III cell sorter; Becton, Dickinson and Company, Franklin Lakes, NJ) to obtain the CD44-knockdown cells MDA/shCD44 and A375/shCD44. As control, cells stably expressing shRNA against firefly luciferase (shLuc) (15) were similarly established (MDA/shLuc and A375/shLuc).

CD44 overexpression

The full-length human CD44 cDNA was reverse transcribed and amplified from MDA-MB-231 mRNA and cloned into the pIRESpuro2 vector (Clontech Laboratories, Inc., Mountain View, CA). The plasmid was transfected into MCF-7 cells using the Nucleofector II. The empty pIRESpuro2 vector was transfected as a control. Colonies resistant to puromycin (0.25 μg/ml) were isolated and cloned (MCF-7/CD44 and MCF-7/EV).

Cell proliferation in monolayers

The cells (1,000 cells/well) were plated in growth medium in 96-well plates and cultured for 24, 48, and 72 hours. Cell proliferation was determined using a Cell Proliferation Reagent
WST-1 (Roche Diagnostics K.K., Tokyo, Japan) according to the manufacturer’s protocol. The absorbance was measured using a microplate reader (Nippon Bio-Rad Laboratories, Tokyo, Japan) at a wavelength of 450 nm. Each assay was performed in quadruplicate and repeated at least three times.

**Tumor sphere formation**

Tumor sphere formation assays were performed as described previously (16). After culture, the number of tumor spheres of >100 μm in diameter was counted by light microscopy. Data are expressed as the number of tumor spheres/well. Each assay was performed in triplicate and repeated at least three times.

**Wound healing assay**

The cells (1 × 10^5 cells/well) were plated in growth medium in 24-well plates and incubated for 24 hours. After confirming the formation of a complete monolayer, the cells were wounded by scratching lines with a standard 200-μl plastic tip. Migration and cell movement throughout the wound area was observed with a phase-contrast microscope after 24 or 48 hours. The percentage of filled wound area was calculated as follows: filled wound area (%) = (original wound area − remaining wound area)/original wound area × 100. Each assay was performed in triplicate and repeated at least three times.

**Cell invasion assay**

Cell invasion assays were performed using 24-well BD Biocoat Matrigel Invasion Chambers (Becton, Dickinson and Company) according to the manufacturer’s instructions. In brief, cells were seeded into upper inserts (2.5 × 10^4 cells/insert) in DMEM supplemented with 0.1% bovine serum albumin (BSA). Outer wells were filled with DMEM containing 10% FBS as the chemoattractant. After 24 hours of incubation, the membranes with invaded cells were stained with hematoxylin, washed, and mounted on slides. The entire membrane with
invading cells was counted by light microscopy. Data are expressed as the number of invaded cells/well. Each assay was performed in triplicate and repeated at least twice.

**Osteoclast differentiation in vitro**

Osteoclast differentiation was examined using mouse bone marrow cultures, as described previously (17). In brief, bone marrow cells harvested from ddY mice (male, 4 weeks old; Japan SLC, Shizuoka, Japan) were cultured in α-minimum essential medium (αMEM; Life Technologies Japan Ltd.) supplemented with 10% FBS in the presence or absence of 4-MU (0.01–0.3 mM) for 6 days. Osteoclast differentiation was induced by 10 μM prostaglandin E2 (PGE2; Cayman Chemical Company, Ann Arbor, MI) or 50 ng/ml macrophage colony-stimulating factor (M-CSF; Kyowa Hakko Kogyo Co., Tokyo, Japan) and 100 ng/ml receptor activator of NF-κB ligand (RANKL; PeproTech EC, London, UK). After culture, the cells were stained with tartrate-resistant acid phosphatase (TRAP) using a commercial kit (Sigma-Aldrich). The TRAP-positive multinucleated (≥3 nuclei) cells in each well were counted by light microscopy. Data are expressed as the number of osteoclasts/well. Each assay was performed in quadruplicate and repeated at least three times.

**Microarray analysis**

Total RNA was extracted using a High Pure RNA Isolation Kit (Roche Diagnostics K.K.). The RNAs were subjected to microarray full service (Hokkaido System Science Co., Ltd., Hokkaido, Japan) and assessed using a Whole Human Genome Oligo Microarray Kit Ver2.0 GeneChip (Agilent Technologies, Inc., Santa Clara, CA). Microarray data were deposited at the National Center for Biotechnology Information Gene Expression Omnibus with the accession number GSE45613.

**Reverse transcriptase-polymerase chain reaction (RT-PCR)**

RT-PCR was performed as described previously (17). Primer sequences were as follows:
HAS1 (270 bp), ACTCGGACACAAGTTGGAC/GGTTGTACCAGGCCTCAAGA; HAS2 (245 bp), AGAGCACTGGGACGAAATGT/ATGCACCTGAACACACCCAAA; HAS3 (297 bp), GTCAGTGGTACGGGTTTCT/AGGCCAATGAAGTTTCACCAC; glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 415 bp), CATGGAGAAGGCTGGGGCTC/CACTGACACGTTGGCAGTGG. The primer sequences for CD44 have been described previously (18). The sizes of the fragments were confirmed by reference to a 100-bp DNA ladder.

**Real-time RT-PCR**

Real-time RT-PCR was performed using a One Step SYBR PrimeScript PLUS RT-PCR Kit (Takara Bio Inc.) with a StepOnePlus Real-Time PCR system (Life Technologies Japan Ltd.). Primer sequences were as follows: HAS1, TGCTCAGCATGGGTTATGC/AGGGCGTCTCTGAGTAGCAG; HAS2, CTCCGGGACCACACAGAC/TCAGGATACATAGAAACCTCTCACA; HAS3, ACCATCGAGATGCTTTGAGT/CCATGAGTCGTACTTGTTGAGG; hyaluronidase-1 (HYAL1), CACGACAAACCACTTTCTGC/CCCAGAGCACCACTCCAG; HYAL2, CTTCAACAGGACCACCTACA/TCACCCCAGAGGATGACAC; HYAL3, AGGCCCTCTGAGATCATGTG/CCCTAACTAGCTGGAACCTGAC; HYAL4, GCACCATAGGAAAGTGCTG/TGCTTCACTTTTGACTAGG; HYAL pseudogene 1 (HYALP1), ATCAAGAGCCAGCCTTTCAA/CGTGAAGGAGCAGCATACT; sperm adhesion molecule 1 (SPAM1), GCACCTCATAAGGTCTTTCA/TTAGTATGTTATCAAGGCAAGATT; GAPDH, AGCCACATCGCTAGACAC/GCCCAATACGACCAAATCC. Melting curve analysis was performed to determine the melting temperature of the amplified products and to exclude undesired primer dimers. Each sample was run at least in triplicate. Quantification was normalized using GAPDH as a reference gene. Expression levels of the specific genes are indicated as fold-changes compared with the controls. Each assay was performed in
triplicate and repeated at least three times.

**ELISA**

Cells (5 × 10⁴ cells/well) were plated in 24-well plates. At near confluence, the medium was changed to fresh growth medium (500 μl/well), and the conditioned medium was collected after 24 hours. HA concentrations were determined by ELISA (Biotech Trading Partners, Inc., Encinitas, CA) according to the manufacturer’s instructions. Data are expressed as HA produced (ng/10⁵ cells/24 hours). Each assay was performed in quadruplicate and repeated at least twice.

**Animal experiments**

Tumor formation in the orthotopic sites and metastases to bone and lung were determined, as described previously (17, 19). For the inoculation of MCF-7 cells, mice received one 60-day-release pellet containing 0.72 mg 17β-estradiol (Innovative Research of America, Sarasota, FL) 2 days before the cell inoculation. The number of mice used in each experiment is indicated in the figures. 4-MU (400 mg/kg/0.1 ml water with 1% arabic gum) was administered daily by oral gavage from days 1–27, and mice were sacrificed on day 28 (20). All animal experiments were reviewed by the Animal Management Committee of Matsumoto Dental University.

**Histological and histomorphometric analyses**

Paraffin sections were prepared by conventional methods. Histomorphometric analysis of the tumor burden in bone and lung and the number of osteoclasts at the tumor–bone interface was performed as described previously (17, 19). Data are expressed as tumor area (mm²), tumor area/total area (%), and the number of osteoclasts/mm bone surface (N. Oc/BS), respectively.
**Immunohistochemistry and immunofluorescence**

**Immunohistochemistry:** Immunohistochemical staining for CD44 was performed using a Histofine Simple Stain Kit (Nichirei Biosciences Inc., Tokyo, Japan) according to the manufacturer's protocol. Chromogen was developed using a DAB Liquid System (Dako Japan Inc., Kyoto, Japan). The slides were counterstained with hematoxylin.

**Immunofluorescence:** Immunofluorescence staining for CD44 and HA was performed as described previously (21). Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI).

**Statistical analysis**

Data are expressed as mean ± SEM. The data were analyzed by one-way ANOVA followed by Tukey–Kramer tests for determining differences between the groups. Student’s t-test was performed for comparing two groups. P values of <0.05 were considered significant.
Results

Expression of CD44 in bone metastatic cancer cell lines

We firstly examined CD44 expression in human cancer cell lines. Flow cytometric analysis demonstrated that CD44 was highly expressed in MDA-MB-231, A375, PC-3, and HARA-B cells (Supplementary Figure S1A - D), all of which have been shown to frequently metastasize to bone when inoculated into mice (17, 22-24). MCF-7 cells, which possess less bone metastatic potential (25), showed lower CD44 expression (Supplementary Figure S1E).

Effects of CD44 knockdown on cancer cells in vitro

To examine the roles of CD44 in cancer metastasis, we established MDA-MB-231 human breast cancer and A375 human melanoma cell clones stably expressing shRNA against CD44 (MDA/shCD44 and A375/shCD44). Flow cytometry confirmed that CD44 knockdown markedly decreased CD44 expression (Supplementary Figure S2A, B). Although monolayer cell proliferation was unchanged (data not shown), tumor sphere formation in suspension cultures was significantly reduced in MDA/shCD44 and A375/shCD44 cells compared with the control MDA/shLuc and A375/shLuc cells (Figure 1A, B). Cell migration and invasion determined by wound healing assays and matrigel invasion assays, respectively, were also suppressed by CD44 knockdown (Figure 1C - F). Conversely, overexpression of CD44 in MCF-7 cells (MCF-7/CD44; Supplementary Figure S2C) exhibited increased tumor sphere formation and cell migration (Supplementary Figure S3A, B).

Effects of CD44 knockdown on tumorigenic and metastatic potential of cancer cells in nude mice

Next, we examined the effects of CD44 knockdown on tumorigenicity and metastasis in vivo. Tumor formation in the orthotopic mammary fat pad was markedly inhibited in mice injected with MDA/shCD44 clones compared with those injected with the control cells (Figure 2A).
A375/shCD44 clones produced similar results (Figure 2B). In contrast, injections of MCF-7/CD44 clones resulted in enhanced tumor formation (Supplementary Figure S3C). More importantly, intracardiac inoculations of MDA/shCD44 and A375/shCD44 cells developed reduced bone metastases (Figure 2C - F). Of note, the number of osteoclasts decreased significantly in the bone metastases of MDA/shCD44 (Figure 2G, H) and A375/shCD44 cells (Figure 2I).

**CD44 expression and HA production**

To further investigate the underlying mechanisms by which the CD44 knockdown inhibited tumorigenesis and bone metastasis, the gene expression profiles of MDA/shCD44 clones were comprehensively analyzed by microarray analysis. Of the three HAS isoforms analyzed, mRNA expression of HAS2 was decreased in MDA/shCD44 cells (Figure 3A), which was confirmed by real-time RT-PCR analysis (Figure 3B). Expression of the hyaluronidases HYAL1, HYAL2, HYAL3, HYAL4, HYALP1, and SPAM1 was unchanged in MDA/shCD44 cells compared with MDA/shLuc cells (Figure 3A). RT-PCR analysis demonstrated that HAS2 was the most highly expressed isoform in MDA/shLuc (Figure 3C) and the parental MDA-MB-231 cells (data not shown). In line with these results, HA production in the conditioned medium was significantly reduced in MDA/shCD44 cells (Figure 3D). HAS2 mRNA expression and HA production were also decreased in A375/shCD44 cells compared with A375/shLuc cells (Figure 3B, D). In A375/shLuc cells, HAS3 is expressed at a level equivalent to HAS2 (Figure 3C) and and the expression was not changed in A375/shCD44 cells (data not shown). Flow cytometric analysis showed that cell surface-associated HA was markedly decreased in MDA/shCD44 cells (Supplementary Figure S4A). The association of HA with the cell surface also correlated with CD44 expression in A375/shCD44 and MCF7/CD44 cells (Supplementary Figure S4B, C).

We then examined the localization of HA in bone metastases. Immunohistochemical examination demonstrated that HA was much more abundantly expressed in the metastatic
lesions of MDA/shLuc and A375/shLuc cells compared with the surrounding bone marrow tissues (Figure 4A, Supplementary Figure S5A). HA primarily localized in the intercellular spaces and frequently co-localized with CD44 expressed by cancer cells (Figure 4B, Supplementary Figure S5B). In contrast, the localization of HA was reduced in the bone metastases of MDA/shCD44 and A375/shCD44 cells, and co-localization with CD44 was rarely observed (Figure 4B, Supplementary Figure S5B).

**Effects of the HA synthesis inhibitor on bone metastasis**

The involvement of the CD44–HA axis in the development of bone metastasis was finally examined using 4-MU, an inhibitor of HA synthesis (26). 4-MU decreased monolayer cell proliferation and tumor sphere formation of parental MDA-MB-231 and A375 cells in a dose-dependent manner (Figure 5A, B, Supplementary Figure S6A, B). Real-time RT-PCR analysis showed that 4-MU downregulated mRNA expression of CD44 and HAS2 (Figure 5C, Supplementary Figure S6C) but did not affect that of HAS3 (data not shown) both in MDA-MB-231 and A375 cells. 4-MU also suppressed PGE2- and RANKL-induced osteoclast-like cell formation in bone marrow cultures (Figure 5D, Supplementary Figure S7). Moreover, 4-MU significantly inhibited bone metastases of MDA-MB-231 cells with reduced number of osteoclasts (Figure 5E - G). Bone metastases of A375 also tended to be smaller in the 4-MU-treated group but the difference was not statistically significant (Supplementary Figure S6D).
Discussion

CD44 has been implicated in various cancer phenotypes, including enhanced cell proliferation, migration, invasion, and metastasis (2). Consistent with this, our results showed that CD44 knockdown in cancer cells suppressed cell migration and invasion \textit{in vitro} and that CD44 overexpression increased migration and invasion. This suggests that CD44 increases bone metastasis by enhancing migration and invasion of cancer cells. However, it is unlikely that these metastasis-promoting effects are specific for bone metastasis. In fact, lung metastasis was also reduced in MDA/shCD44 clones (Supplementary Figure S8).

We previously demonstrated that CD44 is expressed on osteoclasts and their precursor cells (27). Studies using CD44-deficient mice have shown that CD44 expression in osteoclast lineage cells has inhibitory effects on osteoclast formation and function (28, 29). However, the roles of CD44 expressed by other types of cells in bone, including metastatic cancer cells, have not yet been determined. Here, we demonstrated that the number of osteoclasts decreased significantly in bone metastases of MDA/shCD44 and A375/shCD44 cells. On the other hand, HA has been shown to increase receptor activator of NF-κB ligand (RANKL) expression in bone marrow stromal cells through CD44 and to enhance osteoclast differentiation and function (30, 31), suggesting that HA has stimulatory effects on osteoclastic cells. In support of this notion, our data show that 4-MU inhibited osteoclast differentiation \textit{in vitro} and decreased osteoclast number in bone metastases of MDA-MB-231 cells. These finding along with our finding that HA production decreased in MDA/shCD44 and A375/shLuc cells suggest that the CD44 expression in cancer cells stimulates osteoclast formation and bone destruction, at least in part, through an HA-dependent mechanism. It is widely recognized that osteoclastic bone destruction plays crucial roles in the development and progression of bone metastases (32); thus, HA-dependent enhanced osteoclastic activity likely exerts the bone metastasis-specific contribution of CD44.

It has been demonstrated that HA is increased in most cancers and that the increase in HA is
associated with tumor malignancy and poor prognosis (11, 12). Our histochemical analysis showed that HA was markedly increased in the bone metastatic lesions compared with surrounding normal tissues. Of interest, in culture, cancer cells synthesize cell surface-associated HA, whereas the corresponding normal cells secrete most of the HA into the medium (33). Considering that HA is a major ligand for CD44 (1, 2), HA retention on the surface of cancer cells is possibly mediated by CD44. In agreement with this, FACS analysis showed that cell surface-associated HA was decreased in the CD44-knockdown cells and increased in the CD44-overexpressing cells. Furthermore, immunohistochemical examination demonstrated that HA and CD44 frequently co-localized in the bone metastases of MDA/shLuc and A375/shLuc cells, whereas co-localization was markedly decreased in the bone metastases of MDA/shCD44 and A375/shCD44 cells. This CD44-mediated capture may increase the local concentration of HA in bone metastases.

Hematopoietic stem cells (HSCs) synthesize HA, and HA expression correlates with HSC migration to the endosteal niche (34). Importantly, Avigdor et al. showed that HSC homing was blocked by anti-CD44 antibodies, soluble HA, and treatment with hyaluronidase (35). These results suggest that the CD44–HA interaction plays a critical role in stem cell homing to bone marrow. Since HA has also been identified as a component of the cancer stem cell niche (11), it is possible that the CD44–HA interaction facilitates tumor cell arrest and colonization in bone, leading to an increase in the development of bone metastases.

In the present study, microarray analysis revealed that CD44 knockdown downregulates HAS2 mRNA expression, leading to the reduction in HA production. It has also been shown that downregulation of HAS2 reduces CD44 expression in cancer cells (36, 37). Furthermore, consistent with a previous report (38), we showed that the inhibition of HASs by 4-MU suppresses CD44 expression. It is possible that there is a reciprocal regulatory mechanism for the expression of CD44 and HAS2, although the precise mechanism is yet to be determined. On the other hand, HAS3 expression was not affected both by CD44 knockdown and 4-MU treatment in MDA-MB-231 and A375 cells, which has also been reported in other
cancer cells (39). Since A375 cells express a higher level of HAS3 than MDA-MB-231 cells, the inhibitory effect of CD44 knockdown on HA production was moderate compared to MDA-MB-231 cells. These findings provide a plausible explanation for why 4-MU exhibited less effect on the bone metastases of A375 cells. This HA dose-related difference between MDA-MB-231 and A375 cells may also suggest the importance of HA-CD44 interaction on bone metastases.

Several markers have been proposed for identifying CSCs. CD44 is one of the most frequently observed markers and is expressed in stem cells of various cancers, including that of breast, bladder, colon, stomach, head and neck, liver, ovary, pancreas, and prostate cancers and melanoma (2, 3). Our results showed that CD44 downregulation decreased and CD44 upregulation increased tumor sphere formation in vitro and tumor formation in nude mice. These data suggest that CD44 is not just a marker but confer functional properties to CSCs.

Evidence is accumulating that CSCs contribute to the metastatic dissemination of solid tumors. In breast and some other types of cancers, CD44⁺CD24⁻/low cells have been described to possess CSC-like properties (3, 40, 41). A high percentage of CD44⁺CD24⁻/low tumor cells in primary breast cancers has been shown to correlate with the presence of bone metastases (8). It has also been demonstrated that the majority of tumor cells in early bone marrow metastases express the CD44⁺CD24⁻ phenotype (9). Herein, MDA-MB-231 and A375 cells were CD44⁺CD24⁻ and CD44 knockdown markedly suppressed the bone metastases in nude mice without affecting CD24 expression (Supplementary Figure 9A, B). Furthermore, MCF-7 cells, which are CD44⁻/lowCD24⁻ (Supplementary Figure 9C), exhibit low bone metastatic ability. These results suggest that CSCs possess high bone metastatic potential. In contrast, our previous study using the side population (SP) of MDA-MB-231 cells showed that bone metastatic potential was not enhanced in SP cells compared with non-SP cells (16). SP cells are identified as a tail of cells that are dimly stained with Hoechst 33342, a DNA-binding fluorescent dye, when examined by dual-wavelength flow cytometry (16).
Since their original discovery, SP cells have been identified in various normal and malignant tissues and recognized as a stem cell-enriched population (42). Our previous results showed that the SP of MDA-MB-231 cells exhibit some CSC-like properties, such as enhanced tumor sphere formation in culture and increased tumor growth in the mammary fat pads of nude mice, but that CD44 expression is not increased in SP cells (16). Thus, CD44 may be the critical determinant of the development of bone metastases by CSCs. However, of note, CD44 overexpression in MCF-7 cells did not increase bone metastases, and the bone metastatic potential of MCF-7 cells remained low (data not shown), suggesting that CD44 expression is necessary but not sufficient for the development of bone metastases.

The CD44 gene contains 20 exons, 10 of which are variant exons (1, 2). Transcripts are alternatively spliced in various combinations, generating many splice variants (CD44v). The standard form of CD44 (CD44s), which lacks all variant exons, is the smallest and most widely expressed isoform. The insertion of variant exons alters the signaling properties of CD44 by providing additional binding domains for molecules other than HA, and the expression of CD44v has been shown to be associated with tumor progression (1, 2). However, in the cancer cells employed in this study, CD44s is the dominant isoform (Supplementary Figure S10), suggesting that CD44s, but not CD44v, made a major contribution to the development of bone metastasis.

In conclusion, our results suggest that CD44 expression in cancer cells contributes to the promotion of bone metastases by enhancing tumorigenicity, cell migration and invasion, and HA production. Our results also suggest the possible involvement of CD44-expressing cancer stem cells in the development of bone metastases. Therefore, disruption of CD44–HA interaction is a potential target for therapeutic intervention for bone metastases.
References


Figure legends

Figure 1 Phenotypes of MDA/shCD44 and A375/shCD44 clones in vitro
(A, B) Tumor sphere formation of MDA/shCD44 (A) and A375/shCD44 (B) in suspension cultures. Representative microscopic images are shown on the left (scale bar = 500 μm). Data are expressed as the number of tumor spheres/well. *Significantly different from MDA/shLuc or A375/shLuc (p < 0.05).
(C, D) Cell migration of MDA/shCD44 (C) and A375/shCD44 (D) determined by the wound healing assay. Representative microscopic images at 0 and 24 hours after wounding are shown on the left (scale bar = 500 μm). Data are expressed as filled wound area (%). *Significantly different from MDA/shLuc or A375/shLuc (p < 0.05).
(E, F) Cell invasion of MDA/shCD44 (E) and A375/shCD44 (F) determined by the matrigel invasion assay. Data are expressed as the number of cells invaded/well. *Significantly different from MDA/shLuc or A375/shLuc (p < 0.05).

Figure 2 Tumorigenicity and bone metastatic potential of MDA/shCD44 and A375/shCD44 clones in nude mice
(A) Tumor formation of MDA/shCD44 in the orthotopic mammary fat pads of nude mice. Data are expressed as tumor volume (mm³; n = 5/group). *Significantly different from MDA/shLuc (p < 0.05).
(B) Subcutaneous tumor formation of A375/shCD44 in nude mice. Data are expressed as tumor volume (mm³; n = 5/group). *Significantly different from A375/shLuc (p < 0.05).
(C) Representative histological views of bone metastases of MDA/shCD44. The sections were stained with hematoxylin and eosin (H-E, left panels; T, tumor; BM, bone marrow) and immunohistochemically for CD44 (right panels). Scale bar = 500 μm.

(D) Histomorphometric analysis of tumor burden in bone of MDA/shCD44. Data are expressed as tumor area (mm²; n = 9/group). *Significantly different from MDA/shLuc (p < 0.05).

(E) Representative histological views of bone metastases of A375/shCD44. The sections were stained with H-E (T, tumor; BM, bone marrow; scale bar = 500 μm).

(F) Histomorphometric analysis of tumor burden in bone of A375/shCD44. Data are expressed as tumor area (mm²; n = 6/group). *Significantly different from A375/shLuc (p < 0.05).

(G) Representative histological views of bone metastases of MDA/shCD44 stained for tartrate-resistant acid phosphatase (TRAP) (T, tumor; scale bar = 100 μm).

(H) Histomorphometric analysis of osteoclast numbers in the bone metastases of MDA/shCD44. Data are expressed as the number of osteoclasts/mm at the tumor–bone interface (N. Oc/BS; n = 8/group). Since the tumor burden with MDA/shCD44 injections was small, the data from MDA/shCD44-2 and MDA/shCD44-3 injections were combined and presented as MDA/shCD44. *Significantly different from MDA/shLuc (p < 0.05).

(I) Histomorphometric analysis of osteoclast number in bone metastases of A375/shCD44. Data are expressed as the number of osteoclasts/mm at the tumor–bone interface (N. Oc/BS; n = 7/group). Since the tumor burden with A375/shCD44 injections was small, the data from A375/shCD44-2
and A375/shCD44-3 injections were combined and presented as A375/shCD44. *Significantly different from A375/shLuc (p < 0.05).

Figure 3  Hyaluronan synthases (HASs) expression and hyaluronan (HA) production in MDA/shCD44 and A375/shCD44 cells
(A) Expression of HASs and HYALs in MDA/shCD44 determined by cDNA microarray analysis. Raw data are expressed on the left, and the fold-changes compared to MDA/shLuc are shown on the right.
(B) Relative HAS2 mRNA expression in MDA/shCD44 (left) and A375/shCD44 (right) determined by real-time RT-PCR. Data are expressed as fold-changes compared to MDA/shLuc or A375/shLuc. *Significantly different from MDA/shLuc or A375/shLuc (p < 0.05).
(C) mRNA expression of HASs in MDA/shLuc (top) and A375/shLuc (bottom) determined by semi-quantitative RT-PCR analysis.
(D) HA production by MDA/shCD44 (left) and A375/shCD44 (right) in the culture medium determined by ELISA. Data are expressed as HA produced (ng/10^5 cells/24 hours). *Significantly different from MDA/shLuc or A375/shLuc (p < 0.05).

Figure 4  Localization of HA and CD44 in bone metastases of MDA/shCD44 cells
(A) HA expression in bone metastasis of MDA/shLuc and surrounding bone marrow (BM). T, tumor; scale bar = 100 μm.
(B) Expression and co-localization of HA and CD44 in the bone metastases. T, tumor; scale bar = 20 μm.

Figure 5  Effects of 4-MU on bone metastases of parental MDA-MB-231 cells
(A) Effects of 4-MU on MDA-MB-231 cell proliferation in monolayer cultures as determined by the WST-1 assay. Data are expressed as the absorbance at 450 nm. *Significantly different from the control (p < 0.05).

(B) Effects of 4-MU on tumor sphere formation of MDA-MB-231 cells in suspension cultures. Data are expressed as number of colonies/well. *Significantly different from the control (p < 0.05).

(C) Effects of 4-MU on mRNA expression of CD44 and HAS2 in MDA-MB-231 cells. Data are expressed as fold-changes compared to the control. *Significantly different from the control (p < 0.05).

(D) Effects of 4-MU on PGE2-induced osteoclast formation in bone marrow cultures. Representative microscopic views of the osteoclast-like cells are shown on the left (TRAP staining; scale bar = 200 μm). Data are expressed as number of osteoclasts/well. *Significantly different from PGE2 alone (p < 0.05).

(E) Representative histological views of bone metastases of MDA-MB-231 cells treated without (left) or with (right) 4-MU. The sections were stained with H-E (T, tumor; BM, bone marrow; scale bar = 500 μm).

(F) Histomorphometric analysis of tumor burden in bone. Data are expressed as tumor area (mm²; n = 7/group). *Significantly different from the control (p < 0.05).

(G) Histomorphometric analysis of osteoclast numbers in the bone metastases of MDA-MB-231 cells. Data are expressed as number of osteoclasts/mm at the tumor-bone interface (N. Oc/BS; n = 7/group). *Significantly different from the control (p < 0.05).
Figure 1
Figure 2

A. Tumor volume (mm$^3$) over time for shLuc and shCD44-2 versus shCD44-3.

B. Tumor volume (mm$^3$) over time for shLuc, shCD44-2, and shCD44-3.

C. H&E and CD44 staining of tumors for shLuc, shCD44-2, and shCD44-3.

D. Tumor area comparison for shLuc and shCD44.

E. Tumor area comparison for shLuc, shCD44-2, and shCD44-3.

F. Tumor area comparison for shLuc and shCD44.

G. Bone metastasis staining for shLuc and shCD44.

H. N. OC/BS comparison for shLuc and shCD44.

I. N. OC/BS comparison for shLuc and shCD44.

Figure 2

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Figure 3
**Figure 4**

Panel A: HA/DAPI staining showing the distribution of HA and DAPI in bone marrow (BM) and tumor (T) regions.

Panel B: Fluorescence images comparing shLuc and shCD44-2 conditions for HA, CD44, DAPI, and Merge (Bone and T regions).
Figure 5
Cancer Stem-like Cell Marker CD44 Promotes Bone Metastases by Enhancing Tumorigenicity, Cell Motility and Hyaluronan Production

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