Gut-derived serotonin induced by depression promotes breast cancer bone metastasis through the RUNX2/PTHrP/RANKL pathway in mice

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Abstract. Breast cancer metastasizes to the bone in a majority of patients with advanced disease resulting in bone destruction. The underlying mechanisms are complex, and both processes are controlled by an interaction between locally and systemically derived signals. Clinically, breast cancer patients with depression have a higher risk of bone metastasis, yet the etiology and mechanisms are yet to be elucidated. MDA-MB-231 breast cancer cells were used to establish a bone metastasis model by using intracardiac injection in nude mice. Chronic mild stress (CMS) was chosen as a model of depression in mice before and after inoculation of the cells. Knockdown of the RUNX-2 gene was performed by transfection of the cells with shRNA silencing vectors against human RUNX-2. A co-culture system was used to test the effect of the MDA-MB-231 cells on osteoclasts and osteoblasts. RT-PCR and western blotting were used to test gene and protein expression, respectively. We confirmed that depression induced bone metastasis by promoting osteoclast activity while inhibiting osteoblast differentiation. Free serotonin led to an increase in the expression of RUNX2 in breast cancer cells (MDA-MB-231), which directly inhibited osteoblast differentiation and stimulated osteoclast differentiation by the PTHrP/RANKL pathway, which caused bone destruction and formed osteolytic bone lesions. Additionally, the interaction between depression and breast cancer cells was interrupted by LP533401 or RUNX2 knockdown. In conclusion, depression promotes breast cancer bone metastasis partly through increasing levels of gut-derived serotonin. Activation of RUNX2 in breast cancer cells by circulating serotonin appears to dissociate coupling between osteoblasts and osteoclasts, suggesting that the suppression of gut-derived serotonin decreases the rate of breast cancer bone metastasis induced by depression.

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Abbreviations: CMS, chronic mild stress; RUNX-2, runt-related transcription factor 2; PTHrP, parathyroid hormone-related protein; RANKL, receptor activator for nuclear factor-κB ligand; GDS, gut-derived serotonin; μCT, microcomputed tomography; shRNA, short hairpin RNA; MSCs, mesenchymal stem cells; AP, bone-specific alkaline phosphatase; ALZ, Alizarin Red staining; TRAP, tartrate-resistant acid phosphatase

Key words: depression, serotonin, RUNX2/PTHrP/RANKL, bone metastasis, breast cancer

Introduction

Breast cancer is one of the most common cancers worldwide and tends to metastasize to bone, resulting in osteolysis and skeletal-related events (pain and fracture) in patients. The mechanisms underlying this metastasis are complex and involve both particular characteristics of the breast cancer cells and the bone matrix (soil and seed concept). During metastasis, breast cancer cells possess certain properties that enable them to grow in bone, and the bone matrix provides a suitable microenvironment which facilitates the growth of breast cancer cells. The bone microenvironment facilitates dynamic bone resorption and bone formation which maintains the balance of bone mass. Bone remodeling consists of both resorption by osteoclasts (1-5) and new bone formation by osteoblasts (3,5). The two processes of remodeling are coupled temporally and spatially (1,3,5,6) and are controlled by an interplay between locally and systemically derived signals, such as mechanical strain, growth factors, hormones and other molecules (1,5,6). Breast cancer cells disrupt this normal physiological process and generate factors that directly or indirectly induce the formation of osteoclasts in the bone microenvironment, which in turn releases growth factors from the bone matrix (e.g., TGF-β) that stimulate tumor growth and further osteolysis. This reciprocal interaction between breast cancer cells and the bone microenvironment results in a 'vicious cycle' that increases both bone destruction and the tumor burden (7). The current standard of care for breast

cancer patients (i.e., anti-resorptive therapy) with bone loss due to osteolytic bone metastases has some positive effects, but is not curative with regard to tumor burden (8,9).

Depression is a serious complication in women with breast cancer which is often underestimated with a prevalence varying between 10 and 25%. Depressed individuals display lower bone mineral density (BMD) and higher bone resorption than non-depressed subjects and the underlying mechanism is yet to be elucidated. Research has shown that viscosity of platelet membrane may be a bridge to psychiatric illness (10). Arachidonic acid increases with a decrease in membrane viscosity (11-13), which impairs the capacity of platelet and neuronal serotonin receptors to capture serotonin 5-hydroxytryptamine (5-HT) (14). Serotonin does not cross the blood-brain barrier which creates free serotonin in the circulation and the excess of free serotonin (in depression) can induce osteoporosis (15). The critical step in 5-HT biosynthesis is catalyzed by the rate-limiting enzyme Tph (Tph1 and Tph2). Tph1 is expressed mainly in the gut and pineal gland (16), whereas Tph2 is expressed only in neurons (17). Since most circulating serotonin arises from synthesis in the duodenum by specialized neuroendocrine enterochromaffin cells, several studies have elucidated the effect of gut-derived serotonin on the connection between the brain, bone and intestine (18-20). Thus, in depression, a defect in the uptake of serotonin in platelets and neurons increases circulating serotonin, which influences bone mass and induces osteoporosis.

A recent study found that four 5-HT receptor (5-HTR) subtypes (1A, 1B, 2B and 4) were differentially expressed in human breast cancer (21). In human breast cancer cells, 5-HT induced both parathyroid hormone-related peptide (PTHrP) and the metastasis-associated transcription factor Runx2/Cbfa1 (22). The mammary epithelium possesses a local serotonin signaling system which drives PTHrP expression during lactation and in breast cancer cells which may be a passage of breast communication to bone through serotonergic control of PTHrP (23). Evidence has shown that PTHrP plays an important pathogenetic role in the establishment of osteolytic bone lesions in breast cancer. Inhibition of PTHrP can reduce the development of destructive bone lesions as well as the growth of tumor cells in bone (24,25). This finding that serotonin regulates the behavior of breast cancer cells and bone remodeling balance led us to investigate the possible involvement of depression in the control of breast cancer cell metastasis to the bone.

In the present study, we showed that depression promotes the bone metastasis of breast cancer cells through serotonin signaling. We confirmed that gut-derived serotonin inhibits osteoblast differentiation and activates osteoclast differentiation by stimulating the expression of RUNX2 in breast cancer cells. Thus, activation of RUNX2 in breast cancer cells by circulating serotonin appears to dissociate coupling between osteoblasts and osteoclasts, suggesting that suppression of gut-derived serotonin may decrease the rate of breast cancer bone metastasis induced by depression.

Materials and methods

Intracardiac bone metastasis model. All experimental procedures were approved by the Institution of Animal Care and Use Committee (IACUC) at Chongqing Medical University. All the animals were handled in accordance to the guidelines of IACUC animal usage standards. Female BALB/c nude mice (4-6 weeks old) were purchased from the Laboratory Animal Center (Chongqing Medical University) and quarantined within our facility for 2 weeks prior to the experimentation. Nude mice were group housed in plastic cages (n=5/cage) under standard laboratory conditions with a 12-h dark/12-h light cycle, a constant temperature of 20˚C and humidity of 48%. Nude mice were housed in sterile conditions and fed autoclaved standard chow.

MDA-MB-231 cells used in the present study were gifted from the Key Laboratory for Biochemistry and Molecular Pharmacology of Chongqing Medical University. MDA-MB-231 cells were cultured in 10% fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) with 1% penicillin/streptomycin. Cells were trypsinized at 70-90% confluency, rinsed and re-suspended in cold PBS at 10^7 cells/ml. Athymic nude female mice aged 4-6 weeks were anesthetized and injected in the left cardiac ventricle with 100 μl of the cell suspension (10^6 MDA-MB-231 cells). Bone metastasis was assessed weekly for 4 weeks with Faxitron radiographic imaging (Faxitron MX-20 at 35 kV for 12 sec of exposure).

Chronic mild stress (CMS) and sucrose consumption test. To establish the model of depression, the animals were divided into two matched groups and placed in separate rooms. One group was exposed to an initial 2 weeks of chronic mild stressors and the other was left undisturbed. The stress protocol consisted of one period of intermittent illumination, stroboscopic light, grouping, food or water deprivation; two periods of soiled cage and no stress; three periods of 45˚ box tilting. All the stressors lasted from 10 to 14 h. During the 2 weeks of stress stimulation, a sucrose consumption test was performed (three times in the first week and two times in the last week). After the initial 2 weeks of exposure to stress, the unchallenged and stressed groups were subjected to the next experimental procedure (intracardiac injection). Stress was continued for 4 weeks during the entire period of treatment.

Radiographic analysis. Faxitron was used to quantify the bilateral osteolytic lesions in the humeri, femora and tibiae at end time-point. Presence of tumors within the bones was confirmed with histology, and the lesion area was calculated as the average of total osteolytic area per mouse. Data were double-blinded and calculated by at least two independent researchers.

Microcomputed tomography (μCT) analysis. Tibiae from each animal were dissected, cleaned, fixed for 48 h in 10% formalin/PBS, transferred to 70% EtOH, and then loaded into scanning tubes and imaged (vivaCT 40; Scanco Medical). The scans were integrated into three-dimensional (3-D) voxel images and a Gaussian filter (σ=0.8, support=1) was used to reduce signal noise and a threshold of 300 was applied to all analyzed scans. Scans were carried out at 12-mm resolution (E=70 kVp, I=112 μA). Four hundred transverse slices of the proximal tibia were taken from the growth plate and extended distally except from growth plate, and automated contouring

Table I. Gene-specific primers for human and mouse.

Genes	Primers
BSP (mouse)	5'-AAGAGGAAGAATGAGAACGA-3' 5'-GCTTCTTCTCCGTTGTCTCC-3'
Osteocalcin (mouse)	5'-CTGACCTCACAGATGCCAAG-3' 5'-GTAGCGCCGTGAGTCTGTTC-3'
Collagen 1α1 (mouse)	5'-AGTTTCAGGTCTCTGCAGGT-3' 5'-AACTGGCAAGCAAGGAGACA-3'
RANKL (mouse)	5'-TGTACTTTCGAGCGCAGATG-3' 5'-CCACAATGTGTTGCAGTTCC-3'
Cathepsin K (mouse)	5'-AAGTGGTAAGATGACGGGAC-3' 5'-TCTTCACAATGCCTCCGTTC-3'
Calcitonin receptor (mouse)	5'-CGGACTTTGACACAGCAGAA-3' 5'-GTCACCCTCTGGCAGCTAAG-3'
GAPDH (mouse)	5'-ACCCAGAAGACTGTGGATGG-3' 5'-CACATTGGGGGTAGGAACAC-3'
PTHrP (human)	5'-GGAAGCAACCAGCCCACCAG-3' 5'-ACCGCGTAGCTCAGCAGGAA-3'
RUNX2 (human)	5'-TTTGCACTGGGTCATGTGTT-3' 5'-TGGCTGCATTGAAAAGACTG-3'
GAPDH (human)	5'-CAACGAATTTGGCTACAGCA-3' 5'-AGGGGAGATTCAGTGTGGTG-3'

using voxel counting and sphere-filling distance transformation indices was performed.

Histology. Bones were decalcified in 20% EDTA pH 7.4 at room temperature for 3-4 days, and then dehydrated and embedded in paraffin. Hematoxylin and eosin (H&E) staining was used to quantify tumor burden and tumor number in 5-μm paraffin sections. Tumor number and tumor burden were assessed in the long bones presenting with tumors using the Bioquant system imaging software (Bioquant Image Analysis Co., Nashville, TN, USA) as total tumor area per combined area of the six long bones averaged from three sections per bone.

Analysis of gene expression. Total RNA was isolated with TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and used to generate cDNA templates by reverse transcription (RT) reaction with hexamer and Superscript II RT. The first strand cDNA products were further diluted 5- to 10-fold and used as templates for polymerase chain reaction (PCR). All samples were normalized to the expression level of GAPDH. The primers used in this study are shown in Table I.

Western blotting. Western blotting was performed to examine the protein expression levels *in vitro*. Protein concentration was determined with a protein quantitative analysis kit, and 300 mg of protein was run on a 10% gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and blotted onto PVDF membranes. The membranes were blocked with 5% BSA blocking buffer at room temperature for 1 h, and then probed with the primary antibody at room temperature for 1 h. After washing with Tris-buffered saline with Tween-20 (TBST) three times, the PVDF membranes were incubated with an appropriate secondary antibody (antibodies diluted 1:5,000) at room temperature for 1 h, and then the membranes were washed three times in TBST. Immune complexes were detected using the chemiluminescent method, and immunoreactive bands were quantified using an imaging system (Thermo Fisher Scientific, Rockford, IL, USA).

ELISA. Mice were exposed to isoflurane for 30 sec, and blood was collected by cardiac puncture to measure the serum serotonin levels. Serum collection from all mice was conducted between 9:00 a.m. and 12:00 p.m. Blood was centrifuged at maximum speed at 4˚C for 10 min. The serum supernatant was snap frozen and stored at -80˚C. Serum serotonin levels were quantified by ELISA (serotonin kit; Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions.

Co-culture studies. Boyden chambers (1-μm inserts; BD Biosciences, Bedford, MA, USA) were used to test the influence of MDA-MB-231 cells on osteoclasts. Bone marrow cells were collected from the femur and tibiae of BALB/c female mice (4-6 weeks old). Cells (5,000/well) were plated for 3 days and transferred to the bottom of the chamber with RANKL (5 ng/ml). MDA-MB-231 cells ($1x10^5$ cells/well) with or without serotonin (100 μm; Sigma-Aldrich) were plated in the upper layer for 7 days. Osteoclast formation was monitored by TRAP staining analysis. For osteoblast co-culture studies, MC3T3 cells (5,000/well) were harvested with MDA-MB-231 cells for 3 days. After the addition of serotonin (100 μm) with 50 mg/ml ascorbate and 10 mM β-glycerol phosphate (osteogenic media), the two cell lines were cultured for 14 days. MC3T3 cells were fixed in 2% PFA and stained for AP activity and Alizarin Red staining (ALZ) (Sigma-Aldrich).

shRNA knockdown. MDA-MB-231 cells were transfected with shRNA silencing vectors against human RUNX-2 or a scramble control as previously described (47). After transfection, RT-PCR and western blotting were performed to evaluate RUNX-2-knockdown efficiency.

Statistical analysis. All data are presented as means ± SEM. Statistical analyses were performed using one-way ANOVA for multiple comparisons and the two-tailed Student's t-tests. For all analyses, $P<0.05$ was considered to indicate a statistically significant difference and each assay condition was performed in triplicate. The results were repeated in at least three independent experiments.

Results

Depression promotes breast cancer cell metastasis to the bone and increases osteolytic lesions through gut-derived serotonin. Several processes are involved in the metastasis of breast cancer cells to bone which include cell release from the primary tumor into the bloodstream, localization and proliferation in the bone microenvironment. We used an established model of bone metastasis in which MDA-MB-231 human breast cancer cells were inoculated

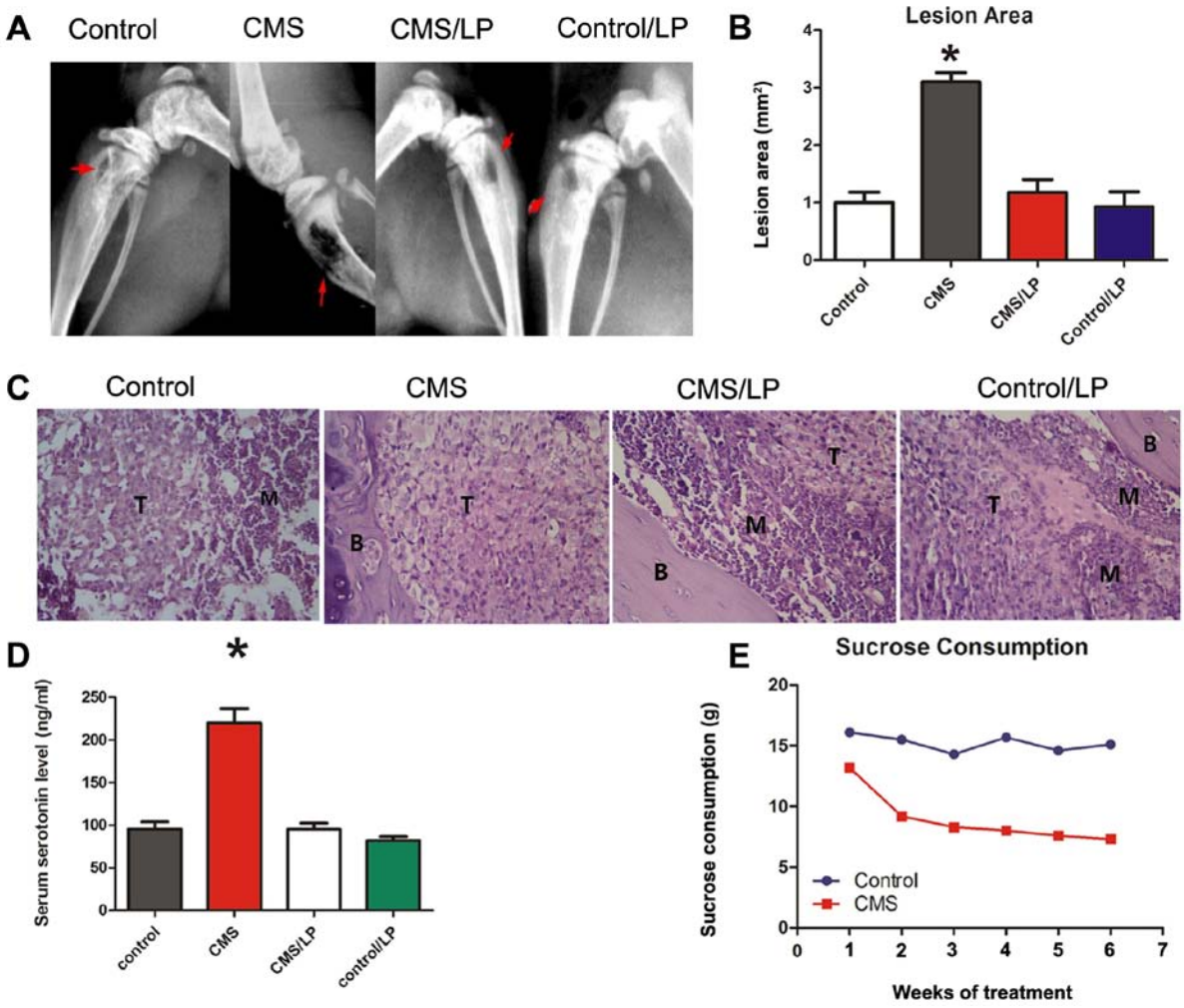

Figure 1. Depression increases bone metastasis of breast cancer through gut-derived serotonin. (A) Faxitron images of limbs 4 weeks after tumor inoculation, showing osteolytic lesions (red arrows). (B) Bone lesion area in the femur, tibiae and humeri of nude mice, measured by Faxitron imaging (n=5/group). (C) Tumor burden is noted in the bones (H&E staining; magnification, x40); T: tumor, B; Bone, M: marrow. (D) Serum serotonin levels (n=5/group). (E) Sucrose consumption after CMS treatment. *P<0.01 vs. control, CMS/LP and control/LP groups. CMS, chronic mild stress; LP, LP533401.

into nude mice by intracardiac injection to elucidate the effect of depression on bone metastasis. CMS was chosen as a model of depression in mice (26-28). In the experimental protocol, nude mice were exposed to an initial 2 weeks of CMS and sucrose consumption was tested before and after intracardiac injection. After 2 weeks of initial exposure to CMS, the intake of sucrose solution was significantly diminished in the stress group compared to the unchallenged animals, indicating a stress-induced decrease in sensitivity to reward (Fig. 1E). After inoculation of breast cancer cells combined with 4 weeks of continuous exposure to CMS, the area of osteolytic lesions increased significantly, measured by Faxitron, compared to the no CMS group (Fig. 1A and B). CMS also significantly increased the number of bone tumors, as measured by histomorphometry (Fig. 1C). LP533401 (Dalton Chemical Laboratories Inc., Toronto, ON, Canada), a small-molecule inhibitor of Tph-1 which is the initial enzyme in gut-derived serotonin (GDS) biosynthesis is currently being tested for the treatment of irritable bowel syndrome with no overt deleterious side effects (29). Oral administration of LP533401 (250 mg/kg/day) for a continuous 6 weeks significantly decreased osteolytic lesions in the CMS group compared to the PBS control (Fig. 1A-C). After 6 weeks of CMS treatment, the level of serum serotonin was significantly increased, which was inhibited by LP533401 (Fig. 1D).

Table II. μCT analysis of long bones from the control, CMS, CMS/LP and control/LP mice.

	Control	CMS	CMS/LP	Control/LP
(BV/TV) (%)	5.78±1.6	2.95±1.2[a]	4.92±1.8[b]	5.34±1.5
Conn.D	26.82±8.9	18.3±6.4[a]	25.43±7.8[b]	26.78±8.4
Tb.Nb	2.01±0.6	1.53±0.3	1.87±0.5	2.12±0.5
Tb.Th	0.046±0.003	0.036±0.004	0.42±0.004	0.48±0.002
Tb.Sp	0.521±0.12	0.652±0.21	0.611±0.17	0.533±0.16

Results were obtained from the tibias of mice. n=5 mice/group. Conn.D, connectivity density; Tb.Nb, trabecular number; Tb.Th, trabecular thickness; Tb.Sp, trabecular spacing. [a]P<0.05 vs. control, [b]P<0.05 vs. CMS/LP group. CMS, chronic mild stress; LP, LP533401.

These findings indicate that the distress induced by CMS in mice increased the incidence of bone metastasis by breast

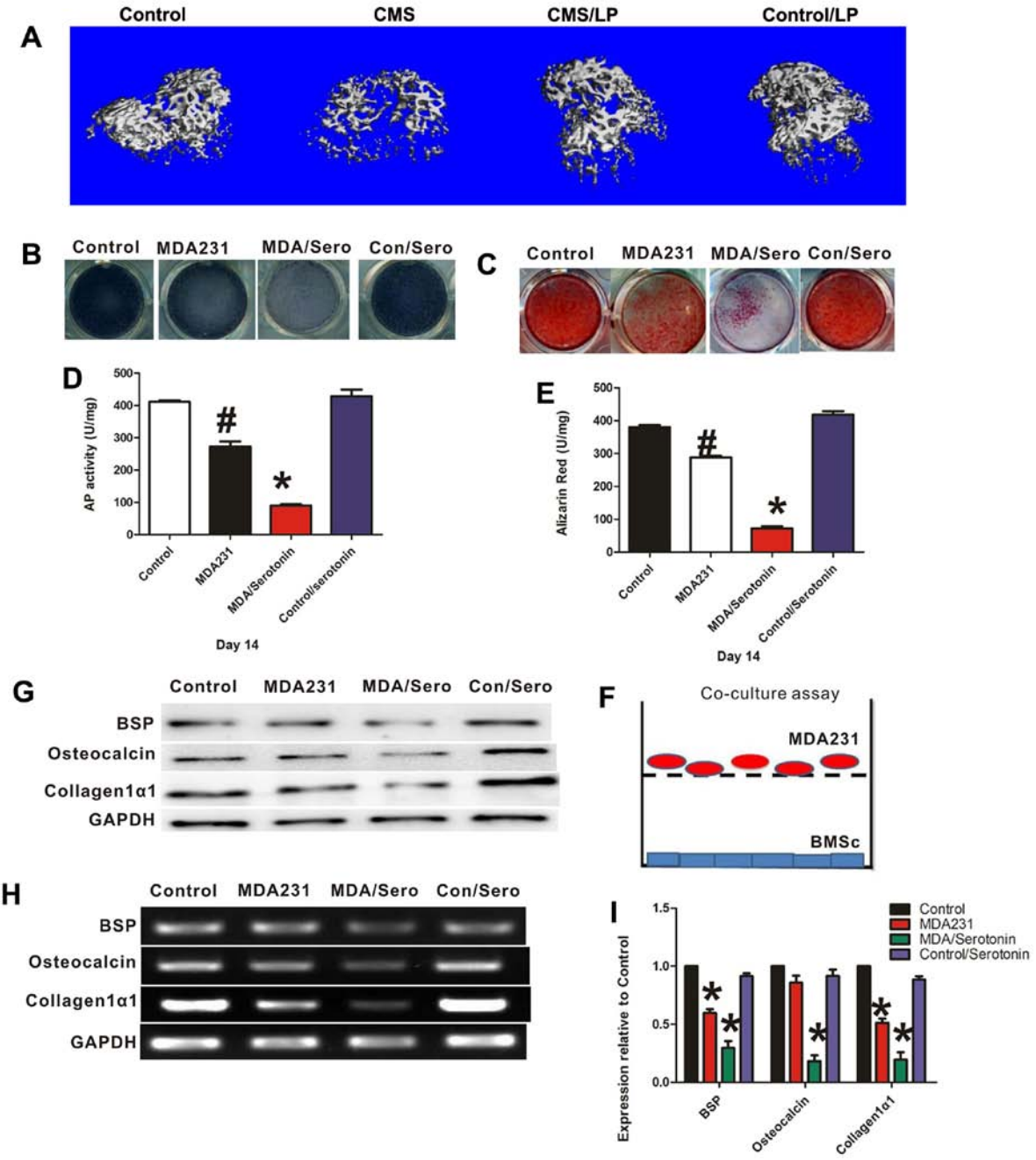

Figure 2. Serum serotonin inhibits osteoblast proliferation and differentiation. (A) Representative 3D images of μCT scan of proximal tibiae. (B and D) Alkaline phosphatase (AP) staining shows inhibition of osteoblast differentiation following the co-culture with MDA-MB-231 cells and addition of serotonin (100 μM).*P<0.01, #P<0.05. (C and E) Alizarin Red staining shows inhibition of osteoblast differentiation following co-culture with MDA-MB-231 cells and addition of serotonin (100 μM). *P<0.01 vs. control and MDA-MB-231, #P<0.05 vs. control. (F and G) Western blotting shows that MDA-MB-231 cells and serotonin inhibited the protein expression of osteoblast differentiation-related genes (BSP, osteocalcin and collagen1α1). (H and I) mRNA expression of osteoblast differentiation-related genes (BSP, osteocalcin and collagen1α1) measured by RT-PCR. *P<0.01 vs. MDA-MB-231 vs. control.

cancer cells, and that blockade of GDS by LP533401 perturbed the bone metastasis, which implicates GDS in the process of breast cancer cell metastasis to bone.

Circulating serotonin increases osteolytic lesions by inhibition of the proliferation and differentiation of osteoblasts. A major mechanism of bone metastasis to the bone of breast cancer cells is decoupled association of bone formation and bone resorption, resulting in bone destruction. To test whether depression has an effect on bone formation in the process of breast cancer bone metastasis, we evaluated *in vivo* and *in vitro* the influence of circulating serotonin on osteoblast differentiation and proliferation. The level of circulating serotonin increased significantly in the CMS treatment group (Fig. 1D), whereas the bone volume and bone formation rate decreased compared to the no CMS group (Fig. 2A and Table II). These data showed that osteoblast proliferation was suppressed in the CMS treatment group. However, oral administration of LP53340 rescued the low bone formation by inhibiting the biosynthesis of GDS. Thus, CMS interferes with bone formation and facilitates the

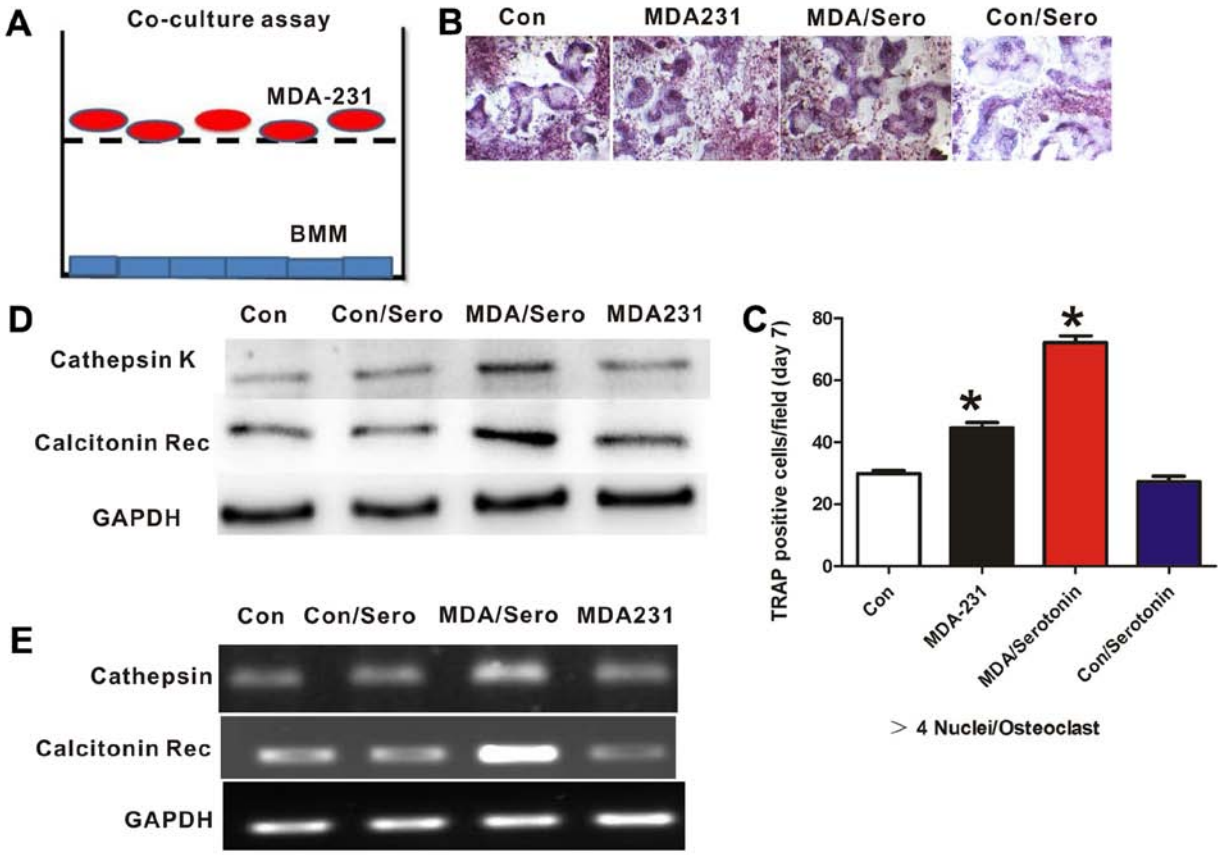

Figure 3. Free serotonin increases osteoclast differentiation. (A) MDA-MB-231-osteoclast co-cultured Transwell assay. (B and C) TRAP staining showed increased osteoclast differentiation when co-cultured with MDA-MB-231 cells and serotonin (100 μM). *P<0.01 vs. MDA-MB-231 vs. control group (magnification, x40 of multinucleated TRAP-positive cells). (D) Western blotting showed that MDA-MB-231 cells added with serotonin promoted protein expression of osteoclast differentiation-related genes (cathepsin K and calcitonin receptor). (E) RT-PCR shows that MDA-MB-231 cells and serotonin promoted protein expression of osteoclast differentiation-related genes (cathepsin K and calcitonin receptor).

bone metastasis of breast cancer cells through inhibition of osteoblast proliferation.

To demonstrate the role of serotonin and breast cancer cells in osteoblast differentiation, Transwell assays were performed. When MDA-MB-231 cells were plated in the Transwell filter with MSCs in the bottom chamber, serotonin treatment (100 μM) significantly decreased the differentiation of osteoblasts, as evidenced by the lower level of bone-specific alkaline phosphatase (AP) (Fig. 2B and D) activity and by decreased mineralization of extracellular matrix, as measured by Alizarin Red staining (ALZ) (Fig. 2C and E). Furthermore, osteoblast-associated gene and protein expression levels of BSP, osteocalcin and collagen type Iα1, were significantly suppressed following treatment with serotonin in the co-culture system (Fig. 2G-I). However, serotonin alone had no direct effect on osteoblast differentiation without co-culture with breast cancer cells. These data showed that serotonin indirectly inhibits osteoblast differentiation by acting on breast cancer cells, which prevents bone formation.

Circulating serotonin increases osteolytic lesions by promoting osteoclast differentiation. To test the effect of serotonin and MDA-MB-231 cells on osteoclast differentiation, we incubated osteoclast progenitors with breast cancer cells in a Transwell system. Osteoclast differentiation was measured by staining cells for TRAP and analyzing osteoclast-specific gene expression. In this co-culture system, serotonin treatment significantly increased osteoclast differentiation, as measured by TRAP activity (Fig. 3B and C). In addition, the expression levels of osteoclast-related gene, cathepsin K, and calcitonin receptor were significantly increased following serotonin treatment (Fig. 3D and E). Thus, free serotonin indirectly promotes osteoclast differentiation by acting on breast cancer cells.

Gut-derived serotonin stimulates the migration of breast cancer cells via RUNX2 signaling. In our previous studies, we found that RUNX2 expression in MDA-MB-231 cells was higher following serotonin treatment (unpublished data). We hypothesized that serotonin might promote bone metastasis by affecting RUNX2 expression in breast cancer cells. To address this question, we established an adenovirus encoding a short hairpin (sh) interfering RNA for human RUNX2. In the experiment, knockdown of RUNX2 by AD-shRUNX2 was effective and specific (>90% reduction in RUNX2 expression by western blotting and RT-PCR) compared to the scramble shRNA control. RUNX2 deficiency reversed the suppression of osteoblast differentiation by serotonin co-culture with breast cancer cells, as evidenced by AP and ALZ (Fig. 4B-E). Furthermore, knockdown of RUNX2 significantly decreased PTHrP expression in the breast cancer cells and RANKL expression in

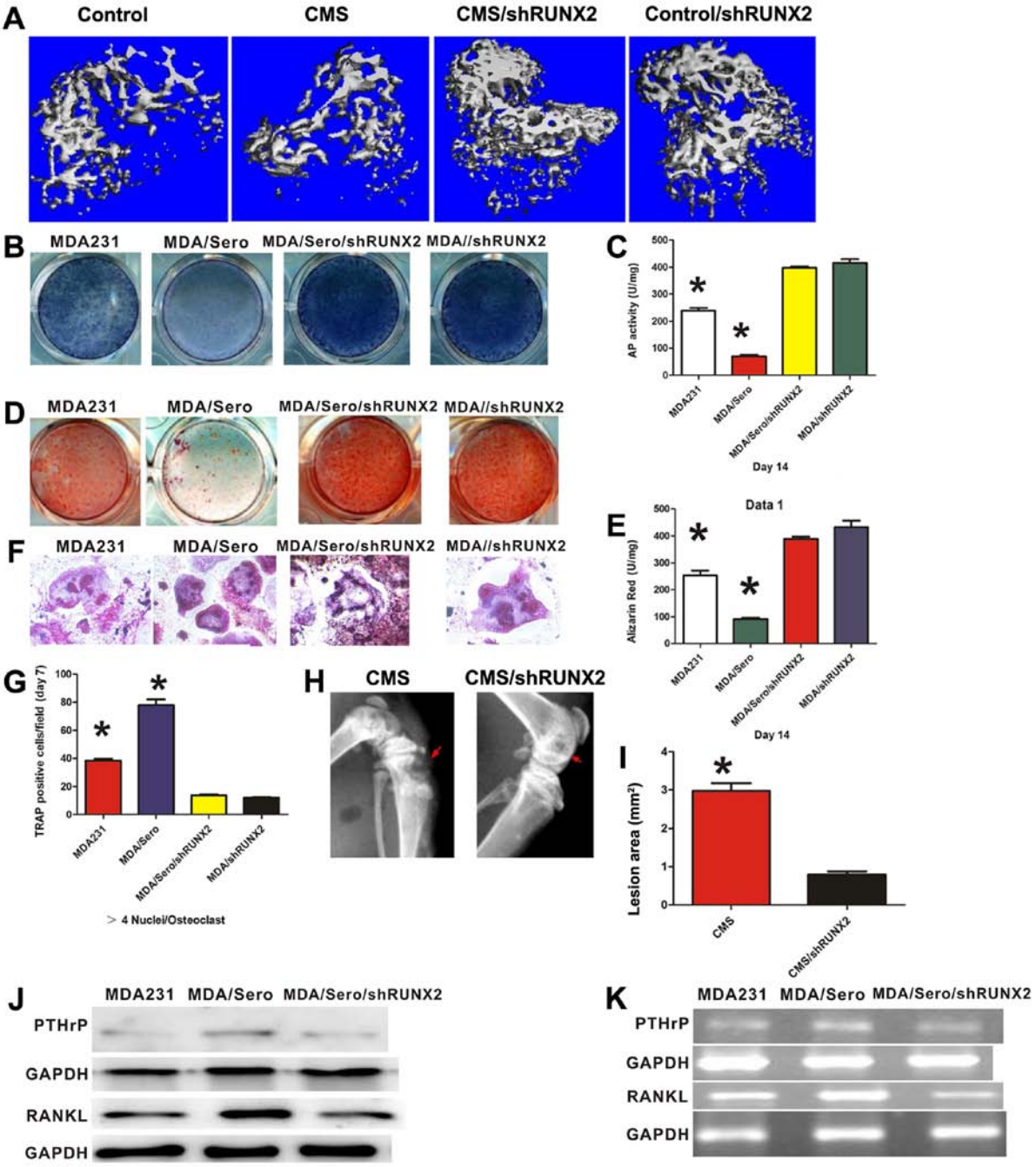

Figure 4. Gut-derived serotonin stimulates the migration of breast cancer cells via RUNX2 signaling. (A) Representative 3D images of μCT scan of proximal tibiae showed that shRUNX2 reversed the low bone formation induced by CMS. (B and D) Alkaline phosphatase staining showed that shRUNX2 reversed the decreased osteoblast differentiation induced by MDA-MB-231 cells with serotonin (100 μM). *P<0.01. (C and E) Alizarin Red staining showed that shRUNX2 reversed the decreased osteoblast differentiation induced by MDA-MB-231 cells with serotonin (100 μM). *P<0.01. (F and G) TRAP staining showed that increased osteoclast differentiation induced by MDA-MB-231 cells and serotonin was reversed by shRUNX2 interference. (magnification, x40 of multinucleated TRAP-positive cells). (H and I) Increased bone lesion area induced by CMS was reversed by shRUNX2 interference (Faxiton image). *P<0.01. (J and K) Western blotting and RT-PCR showed that shRUNX2 reversed the increased PTHrP and RANKL gene and protein expression induced by MDA-MB-231 cells with serotonin (100 μM).

osteoblast cells treated with serotonin (Fig. 4J and K). In the co-cultured system of breast cancer cells and pre-osteoclasts isolated from mouse bone marrow, knockdown of RUNX2 also profoundly decreased the differentiation of osteoclasts, as measured by TRAP staining (Fig. 4F and G). The implication of these findings is that free serotonin disrupted the remodeling process of the bone microenvironment through RUNX2 signaling. To further elucidate the effect of RUNX2 on the bone metastasis of breast cancer cells in depression, we inoculated MDA-MB-231 shRUNX2 and control cells (scrambles) via intracardiac injection in nude mice exposed to CMS to mimic a depressed state. Knockdown of RUNX2 in the MDA-MB-231 cells inhibited bone formation as measured by μCT (Fig. 4A and Table III) and eliminated the effect of CMS on osteolytic lesions as measured by Faxitron (Fig. 4H and I). These results demonstrated that RUNX2 signaling is involved in the regulatory function of depression on breast cancer cell bone metastasis.

Table III. μCT analysis of long bones from control, CMS, CMS/shRUNX2 and control/shRUNX2 mice.

	Control	CMS	CMS/ shRUNX2	Control/ shRUNX2
(BV/TV) (%)	5.44±1.5	2.88±1.3[a]	6.82±1.7[b]	6.18±1.4
Conn.D	27.38±7.1	17.8±4.6[a]	31.89±9.2[b]	34.32±7.4
Tb.Nb	2.33±0.5	1.67±0.4	2.75±0.6	2.88±0.6
Tb.Th	0.043±0.002	0.032±0.001	0.54±0.002	0.58±0.003
Tb.Sp	0.547±0.21	0.678±0.18	0.436±0.12	0.421±0.17

Results were obtained from the tibias of mice. n=5 mice/group. Conn.D, connectivity density; Tb.Nb, trabecular number; Tb.Th, trabecular thickness; Tb.Sp, trabecular spacing. [a]P<0.05 vs. control, [b]P<0.05 vs. CMS group. CMS, chronic mild stress.

Discussion

In the present study, we used both *in vitro and in vivo* models to functionally test the effect of depression on breast cancer-induced bone metastasis. We first established a model of depression by CMS and a breast cancer bone metastatic model by intracardiac injection. Our results demonstrated that CMS significantly promoted osteolytic lesions of breast cancer by inhibiting the proliferation and differentiation of osteoblasts and by activating the differentiation of osteoclasts. Furthermore, gut-derived serotonin signaling participated in the migratory process of breast cancer cells in the state of depression. We confirmed that circulating serotonin directly inhibits osteoblast proliferation and indirectly blunts osteoblast differentiation via RUAN2 signaling in the breast cancer cells and indirectly stimulates osteoclast differentiation by activating the RUNX2-PTHrP-RANKL pathway in the interaction between tumor cells and osteoblasts. Together, our data demonstrated that depression produces a higher level of circulating serotonin, which is essential for the bone metastasis of breast cancer cells, and RUNX2 signaling is the key mechanism in the process of colonization and invasion to the bone microenvironment of tumor cells.

Recent prospective research with mixed types of cancers indicates that depression at the time of diagnosis is associated with an increase in mortality (30,31). Depression is strongly associated with mortality in younger patients with early stage breast cancer (32). Yet, little is known concerning the effect of depression on bone metastasis of breast cancer. Here, we found that depression facilitates the colonization of breast cancer cells in the bone microenvironment and accelerates bone mass destruction in a mouse model. To elucidate the relationship between depression and bone loss, Yirmiya and Bab identified a total of 23 studies and found that depressed individuals had lower BMD and higher bone resorption markers than non-depressed subjects (33). Yadav and Ducy demonstrated that circulating serotonin suppressed osteoblast proliferation by binding to the Htr1b serotonin receptor in bone. Furthermore, LP533401 inhibitor of the initial enzyme of GDS, reversed the effect of serotonin (34). Similar to these observations, our experiments found that the circulating level of serotonin was significantly higher in depressed mice than that in the control group (not depressed). Moreover, high free serotonin is responsible for the inhibition of osteoblast proliferation and this inhibition can be reversed by LP533401, which is a selective Tph1 inhibitor, *in vivo*. More interestingly, serotonin can also suppress osteoblast differentiation when co-cultured with breast cancer cells. One explanation for this is that serotonin has an indirectly regulatory function on osteoblasts by acting on breast cancer. In contrast to a study by Kode *et al* (35), which showed no effect of serotonin on osteoclast differentiation, our studies *in vitro* found that circulating serotonin had a positive effect on osteoclast differentiation by acting on breast cancer cells. These striking findings suggest that depression decouples the remodeling process of the bone microenvironment by elevating the level of free serotonin, which results in metastatic bone loss by inhibiting bone formation and promoting bone resorption. Thus, bone metastasis is generated by both increased activation of osteoclasts and suppression of osteoblasts.

It has been suggested that cancer cells preferentially metastasize to bone due to their ability to express genes that are normally considered bone or bone-related (36). In this process, cancer cells are equipped to home, adhere, survive and proliferate in the bone microenvironment by osteomimetic factors including osteopontin (OPN), osteocalcin, osteonectin, bone sialoprotein, RANKL and PTHrP. Several of these molecules related to the recruitment and differentiation of osteoclasts play prominent roles in the vicious cycle, which can in turn contribute to tumor cell survival, proliferation, adhesion and migration (37). Interestingly, many osteomimetic factors are regulated by the same transcription factor, Runx2. Runx2 is considered to be the major regulator of osteoblast commitment and differentiation (38). RUNX2 is one of the primary transcriptional regulators of new bone formation and skeletal maintenance and ablation of this gene in mice can result in chondrogenic and osteogenic maturation (39,40). Recent studies showed that metastatic breast cancer cells express the transcriptional factor RUNX2, which can inhibit osteoblast differentiation. Moreover, expression of RUNX2 concurrently enhanced osteoclast differentiation in a marrow stromal cell culture (41-43). Consistent with these observations, our findings showed that RUNX2 and RUNX2 target genes were necessary for depression-induced bone metastasis of breast cancer. We observed that RUNX2 knockdown impacted breast cancer cell growth in bone and osteolytic lesions stimulated by depression. We also observed that when co-cultured with breast cancer cells *in vitro*, RUNX2 knockdown eliminated the inhibition of osteoblast differentiation and activation of osteoclast differentiation under a free serotonin condition. These observations showed that tumor cells lacking intact Runx2 function, independent of growth, did not activate local osteoclasts and suppress osteoblasts. Thus, our results support the conclusion that Runx2 activity is required for stimulation of bone metastasis by depression.

Previous studies suggest that production of PTHrP is more common in metastatic breast cancer cells in bone than in the primary tumor, and that PTHrP may be responsible for the local bone destruction occurring in patients with breast cancer, even in the absence of hypercalcemia or increased plasma PTHrP concentrations. The role of PTHrP in bone metabolism is not fully understood, but it is known to cause upregulation

of RANKL and downregulation of OPG, which can enhance osteoclast function leading to bone degradation (44,45). PTHrP, which is secreted by breast cancer cells, can induce osteoclastic bone resorption by stimulating RANKL expression in osteoblasts (46).

Analogous to these observations, our study showed that serotonin treatment increased the expression of PTHrP in breast cancer cells and RANKL in osteoblasts which can be suppressed by RUNX2 knockdown. The data presented here suggest the possibility that PTHrP may be an effector of RUNX2 in bone metastases, as overexpression of PTHrP in breast cancer cells consistent with overexpressed RUNX2 resulted in accelerated bone metastases. The effect of RUNX2 on tumor cells to stimulate PTHrP may result in adverse effects when tumor cells are housed in bone. Tumor cells in the bone microenvironment produce PTHrP, which causes upregulation of RANKL in osteoblasts, thus enhancing osteoclast function leading to bone degradation.

In summary, we used a mouse model of depression to confirm that depression promotes bone metastasis of breast cancer which is associated with decoupled interplay between osteoclasts and osteoblasts. By knockdown of RUNX2, we found that the RUNX2/PTHrP/RANKL pathway plays an important role in breast cancer bone metastasis induced by depression. These findings are significant for the elucidation of the mechanisms of breast cancer bone metastasis and provide insight into the prevention and/or treatment of the osteolysis caused by breast cancer.

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