ITGBL1 Is a Runx2 Transcriptional Target and Promotes Breast Cancer Bone Metastasis by Activating the TGFβ Signaling Pathway

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Abstract

Bone metastasis affects more than 70% of advanced breast cancer patients, but the molecular mechanisms of this process remain unclear. Here, we present clinical and experimental evidence to clarify the role of the integrin β-like 1 (ITGBL1) as a key contributor to bone metastasis of breast cancer. In an in vivo model system and in vitro experiments, ITGBL1 expression promoted formation of osteomimetic breast cancers, facilitating recruitment, residence, and growth of cancer cells in bone microenvironment along with osteoclast maturation there to form osteolytic lesions. Mechanistic investigations identified the TGFβ signaling pathway as a downstream effector of ITGBL1 and the transcription factor Runx2 as an upstream activator of ITGBL1 expression. In support of these findings, we also found that ITGBL1 was an essential mediator of Runx2-induced bone metastasis of breast cancer. Overall, our results illuminate how bone metastasis occurs in breast cancer, and they provide functional evidence for new candidate biomarkers and therapeutic targets to identify risk, to prevent, and to treat this dismal feature of advanced breast cancer. Cancer Res; 75(16); 3302–13. © 2015 AACR.

Introduction

Breast cancer cells preferentially metastasize to the bone, leading to osteolytic lesions. To form visible metastatic bone lesions, breast cancer cells undergo processes, including recruitment to the bone, survival, and clonal expansion in the bone microenvironment, as well as osteoclast activation to resorb the bone matrix (1). Increasing evidence has demonstrated that the ectopic expression of bone-remodeling genes or gene signatures in primary breast cancer cells increases the risk of bone metastasis (2); however, the underlying molecular mechanisms remain largely unknown.

Integrin beta-like 1 (ITGBL1), which was first cloned and characterized from an osteoblast cDNA library (3), encodes a ten integrin EGF-like repeat domain-containing protein (TIED). The TIED1 protein is highly homologous to the N-terminal EGF-like stalk fragment of integrin β (4), but contains neither a transmembrane domain nor an RGD (Arg–Gly–Asp)-binding domain, suggesting that ITGBL1 performs functions distinct from those of integrin. In our previous gene-expression profiling dataset of breast cancer tissues, ITGBL1 was coexpressed with genes encoding proteins involved in bone remodeling and bone metastasis (5). García and colleagues (6) found that ITGBL1 is overexpressed in bone metastatic subclone cells compared with their parental cells and is included in the "osteoblast-like gene expression signature" and "bone metastatic gene signature." These evidences implied that ITGBL1 might contribute to the development of the osteoblast-like (osteomimetic) phenotype and bone metastasis of breast cancer cells. Currently, information regarding the role and molecular mechanism of ITGBL1 expressed by breast cancer cells in bone metastasis remains limited.

Runx2 is a critical transcription factor for osteogenic lineage commitment and bone formation. It switches on the expression of several bone matrix-remodeling genes by binding to the osteoblast-specific cis-acting element 2 (OSE2; refs. 7, 8). Runx2 and its target genes are highly expressed in breast cancer tissues and play pivotal roles in breast cancer bone metastasis (9–12). On the basis of the evidence that ITGBL1 is coexpressed with RUNX2 in breast cancer cells (6) and the fact that there are OSE2 motifs in the ITGBL1 promoter, we hypothesized that ITGBL1 might be a transcriptional target of Runx2 and mediate Runx2-driven bone metastasis.

In this study, we present both in vivo and in vitro evidence to clarify the roles and the underlying molecular mechanisms of ITGBL1 in breast cancer bone metastasis. Moreover, we identify ITGBL1 as a transcriptional target of Runx2, mediating the Runx2-driven bone metastatic potential of breast cancer cells.
**Materials and Methods**

**Patients and tissue samples**

A total of 88 primary breast cancer tissue specimens were collected from breast cancer patients who developed distant metastases within a 5-year follow-up period. Of these patients, 10 patients developed bone-only metastasis, and the other 78 patients suffered other organ metastases with or without bone metastasis. The use of these tissues was approved by the Institutional Review Board and the Research Ethics Committee of Tianjin Medical University Cancer Institute and Hospital (TMUCIH), and written consent was obtained from all participants.

**Cells and treatment**

The human breast cancer MDA-MB-231 and T47D cells, osteoblast-like MG-63 cells, immortalized lung epithelial BEAS-2B cells, and mouse osteoclast precursor RAW 264.7 cells were obtained from the ATCC. To block the TGFβ signaling pathway, cells were treated with 1.0 μmol/L SB-431542 (Santa Cruz Biotechnology) diluted in DMSO or with an equal amount of DMSO as control.

**In vitro chemotaxis assays**

In vitro cell chemotaxis assays were performed using Boyden chamber inserts (BD Biosciences). A total of 2.5 × 10⁴ cancer cells were seeded in the upper chambers and allowed to migrate toward 80% confluent MG-63 or BEAS-2B cells in the bottom chambers for 12 hours (MDA-MB-231) or 30 hours (T47D). Then, the migrated cells were fixed, stained, and counted.

**In vitro osteoclastogenesis assays**

Primary preosteoclasts were isolated from bone marrow cells flushed from the tibias of 6-week-old wild-type Balb/c mice and cultured overnight in αMEM with 10% FBS. A total of 5 × 10⁵ nonadherent cells were plated in a 24-well plate supplemented with 50 ng/mL macrophage colony stimulating factor (M-CSF; PeproTech) for 2 days and then cultured with cancer cell conditioned media (CM) containing 50 ng/mL receptor activator of NFκB ligand (RANKL; PeproTech) for 5 additional days. Osteoclast precursor RAW 264.7 cells were induced for the first 4 days with 50 ng/mL RANKL and then cultured with cancer cell CM containing RANKL for another 3 days. Multinuclear cells that stained positive for tartrate-resistant acid phosphatase (TRAP) were scored as mature osteoclasts.

**Animal experiments**

ITGBL1–GFP-overexpressing MDA-MB-231 cells or control cells were injected into the left cardiac ventricle (1.0 × 10⁶; ref. 13), the cortex of the right tibia (1.0 × 10⁶; ref. 14), or the left lower abdominal mammary fat pad (5.0 × 10⁶) of 5-week-old female SCID mice. The formation of tumors and metastases was assessed and observed by bioluminescence imaging using a Xenogen IVIS 200 Imaging System (Caliper Life Sciences) at weeks 4 and 8. At week 8, the mice were sacrificed to observe osteolytic lesions using an X-ray and SkyScan microCT system (Bruker, Eschborn, GER). Metastases in the lung, liver, and bone were observed by hematoxyl and eosin (H&E) staining. TRAP staining was used to identify the mature osteoclasts in metastatic bone lesions. The animal experiment protocols were approved by the Animal Ethics Committee of TMUCIH.

**Statistical analysis**

Kaplan–Meier survival curves and the log-rank test were used to evaluate the bone metastasis outcomes of patients with different ITGBL1 expression profiles. The Wilcoxon rank-sum test was used to determine the differences in the ITGBL1 expression values among different groups. A repeated measure ANOVA was used to compare the differences in the proliferation capabilities of cancer cells. All other comparisons were determined using the two-tailed Student t test.

**Results**

**ITGBL1** is coexpressed with a set of genes related to bone remodeling and bone metastasis in primary breast cancer tissues

On the basis of our previous gene expression profiling dataset (5) of breast cancer tissues and the GOBO (15) dataset (Fig. 1A), we found that ITGBL1 was coexpressed with a set of genes related to bone remodeling and bone metastasis encoding the osteoblast-specific transcription factor Runx2; osteoblast-specific adhesion molecules OB-Cadherin/CDH11 and integrins (ITG); bone matrix proteins, including collagens (COL), osteoblast-specific factor 2 (OSF-2) and osteoglycin (OGN); and the growth factors TGFβ3 and bone morphogenetic protein 1 (BMP1). These results suggest that ITGBL1 may contribute to the formation of “osteomimetic” breast cancer, which is characterized by the ectopic expression of bone remodeling-related factors and the properties of bone metastasis (16, 17).

Breast cancers with high ITGBL1 expression levels are prone to metastasize to bone

Because of the coexpression of ITGBL1 with a set of genes related to bone remodeling and bone metastasis, we questioned whether ITGBL1 expression is involved in bone metastasis. We used RT-qPCR to quantify the mRNA expression levels of ITGBL1 in 88 primary breast cancers that developed distant metastases within a 5-year follow-up period. The optimized cutoff value was used to group the patients into a low ITGBL1 mRNA group (ITGBL1low) and a high ITGBL1 group (ITGBL1high). The incidence of bone-only metastasis in the ITGBL1high group (17.0%; 9/53) was significantly higher than in the ITGBL1low group (2.3%; 1/35). Patients in the ITGBL1high group had higher risks of bone-only metastasis than patients in the ITGBL1low group [OR, 5.4; 95% confidence interval (CI), 0.7–42.8; P = 0.072; Fig. 1B]. Next, the combined Wang–Minn microarray dataset (18, 19) revealed that the tumors developing bone metastasis expressed significantly higher ITGBL1 mRNA levels than those with non-bone metastases (Supplementary Fig. S1A and S1B). The incidences of both bone metastasis and bone-only metastasis (25.4% and 22.4%, respectively) were significantly higher in the ITGBL1 high group than in the ITGBL1 low group (14.4% and 8.3%, respectively). In addition, patients in the ITGBL1 high group had higher risks of bone metastasis (OR, 1.8; 95% CI, 1.0–3.3; P = 0.033) and bone-only metastasis (OR, 2.6; 95% CI, 1.3–5.0; P = 0.005) than patients in the ITGBL1low group (Fig. 1B and C). Interestingly, we found a negative link between ITGBL1 mRNA levels and non-bone metastases (lung and brain metastases; Fig. 1B and C; Supplementary Fig. S1C–S1E). Taken together, these results provide clinical evidence for the role of ITGBL1 in facilitating the bone-specific metastasis of breast cancer. Moreover, on the basis of the Zhang and colleagues dataset (20), the ITGBL1
mRNA expression levels in bone metastatic tissues were significantly higher than in metastatic tissues of other organs (Fig. 1D). All of the bone metastatic tumor samples (18/18) were sorted into the \( \text{ITGBL1} \) high group, and none of these samples was included in the \( \text{ITGBL1} \) low group (Fig. 1E). Thus, this clinical evidence further suggests that high ITGBL1 expression may help breast cancer cells to survive in the bone microenvironment and facilitate the bone metastasis of breast cancer.

ITGBL1 contributes to the seeding of bone metastases of cancer cells and osteolytic bone lesions

Taking into account of the potential effect of tumorigenicity on the metastatic capability of cancer cells, ITGBL1-overexpressing MDA-MB-231 cells and control cells were injected into the mammary fat pad of mice. We observed a similar tumorigenicity of ITGBL1-overexpressing cells (5/5) with the control cells (5/5), although ITGBL1-overexpressing cells lead to smaller tumors than the control cells (Fig. 2A). Then, considering the high correlation of \( \text{ITGBL1} \) and bone metastasis in clinical breast cancer cases, we speculated that ITGBL1 in breast cancer cells could endow tumor cells with the ability to seed bones as these cells disseminated in the circulation. We inoculated ITGBL1–GFP-overexpressing MDA-MB-231 cells or control cells into the left cardiac ventricle of SCID mice. The incidence of bone metastasis generated by ITGBL1-overexpressing cells was 75% (3/4) at 90 days (Fig. 2B and C), which is higher than the incidences generated by the control cells (0/3; Fig. 2C) and the parental MDA-MB-231 cells (31% at 100 days) reported by Kang and colleagues (21). Moreover, ITGBL1-overexpressing cancer cells led to serious osteolytic bone lesions with a greater number of TRAP\(^+\) osteoclasts, but reduced lung metastasis (1/4; Fig. 2B–E), whereas all mice (3/3) in the control group died of serious lung metastasis within 40 days.
ITGBL1 in Breast Cancer Bone Metastasis

Figure 2.
ITGBL1 enhances breast cancer seeding to the bone. A, tumor incidence and tumor volume in the mammary fat pad following orthotopic injection of ITGBL1-overexpressing MDA-MB-231 cells (n = 5) or control cells (n = 5). B, Kaplan–Meier survival analysis representing the overall survival of mice with intracardiac injection or intratibial injection of ITGBL1-overexpressing MDA-MB-231 cells or control cells. C, table showing the incidence of metastases in the bone, lung, and liver generated by the indicated cells. D and E, ITGBL1-overexpressing MDA-MB-231 cells (n = 4) or control cells (n = 3) were injected into the left cardiac ventricle of SCID mice. The histograms (E) display the quantitative bioluminescence of BM at the limbs and the number of lung metastatic (LM) nodules. F and G, ITGBL1-overexpressing MDA-MB-231 cells (n = 4) or control cells (n = 5) were injected into the cortex of the right tibia of SCID mice (F). The histograms display the quantitative bioluminescence and volume of osteolytic lesions in the right tibia (G). Representative bioluminescence imaging, X-ray and micro-CT imaging, H&E staining, and TRAP staining show the bone, lung metastatic lesions, and activated osteoclasts. T, tumor cells; B, bone tissue; and BM, normal bone marrow; *; P < 0.05; ***, P < 0.001.
ITGBL1 facilitates breast cancer cell recruitment to osteoblasts in vitro

We tested the ability of two ITGBL1 knockdown (KD) MDA-MB-231 subclones (ITGBL1 KD1 and ITGBL1 KD2), two ITGBL1-overexpressing MDA-MB-231 subclones (ITGBL1-1 and ITGBL1-2), ITGBL1-overexpressing T47D cells (ITGBL1), and their respective control cells to migrate toward osteoblast-like MG-63 cells or lung epithelial BEAS-2B cells using a Transwell chemotaxis assay in vitro to investigate whether ITGBL1 facilitates cancer cells homing to bone. As shown in Fig. 3A, ITGBL1 KD reduced the chemotactic migration of MDA-MB-231 cells toward MG-63 cells. Conversely, ITGBL1 overexpression increased the chemotactic migration of both MDA-MB-231 and T47D cells toward MG-63 cells (Fig. 3A). However, ITGBL1 KD increased and ITGBL1 overexpression decreased the chemotactic migration of cancer cells toward BEAS-2B cells (Fig. 3B). Considering the potential role of ITGBL1 in the formation of osteoimmunotropic breast cancer, we speculated that osteoimmunotropic breast cancer with high ITGBL1 expression might adapt to survive in the bone rather than in the lung. Therefore, we assessed the colony formation and proliferation capabilities of ITGBL1 KD and ITGBL1-overexpressing cells, as well as their respective control cells, in CM from MG-63 and BEAS-2B cells, which were used to mimic the bone and lung microenvironment. ITGBL1 KD reduced both of the anchorage-independent and anchorage-dependent survival and proliferation capacity of cancer cells in MG-63 CM, but increased the anchorage-independent survival in BEAS-2B CM (Fig. 3C–F and Supplementary Fig. S2A). In contrast, ITGBL1 overexpression enhanced the survival and proliferation of cancer cells in MG-63 CM and weakened their survival in BEAS-2B CM (Fig. 3C–F and Supplementary Fig. S2A). These results provide in vitro evidence that ITGBL1 plays important roles in cancer cell recruitment to bone and in survival and growth in the bone microenvironment.

To further investigate the molecular mechanisms of ITGBL1 in the bone metastasis of breast cancer cells, we performed gene expression profiling of two ITGBL1 KD subclones, control cells and parental MDA-MB-231 cells by cDNA microarray analysis. Differentially expressed genes with more than 2-fold changes in ITGBL1 KD subclones compared with control cells were identified, including 19 bone metastasis-related genes identified by Kang and colleagues (21), 13 osteoblast-like breast cancer gene markers identified by Bellahcene and colleagues (16), 4 receptors for chemostatic factors, 3 proliferation-related genes, and several genes involved in ECM remodeling and bone metastasis-related signaling pathways (Supplementary Table S2). Then, we demonstrated that ITGBL1 KD reversed the osteoimmunotropic phenotype and further weakened the bone metastatic potential of MDA-MB-231 cells by clustering the expression profiling of ITGBL1 KD subclones, control cells and parental cells based on Bellahcene and colleagues osteoblast-like signature (16) and Kang and colleagues bone metastasis signature (21), as shown in Supplementary Fig. S2B. Using immunoblotting and RT-qPCR, we identified adhesion molecules involved in bone metastasis, including integrin α5 (ITGα5; refs. 22, 23) and CD44 (24), and the osteoimmunotropic markers osteopontin (OPN), bone sialoprotein (BSP), osteonectin (OSN), and OSF-2 that decreased in ITGBL1 KD cells and increased in ITGBL1-overexpressing cells compared with their control cells (Fig. 3G and Supplementary Fig. S2C and S2D). Taken together, these results provide a suitable molecular basis for ITGBL1 activity in promoting the bone metastasis of breast cancer.

ITGBL1 contributes to osteoclastogenesis in the bone microenvironment

Because of the osteolytic characteristics of breast cancer, we further investigated whether ITGBL1 in breast cancer cells contributes to osteoclast formation and differentiation. We isolated primary osteoclasts from bone marrow cells of mice and induced them with cancer cell CM in the presence of RANKL. No significant changes in the number of TRAP+ cells were observed in the primary osteoclast cultures with cancer cell CM with different ITGBL1 expression levels. However, TRAP+ osteoclasts induced by the CM from ITGBL1 KD cells were significantly smaller than those induced by the CM from control cells, and the CM of ITGBL1-overexpressing cells induced larger TRAP+ osteoclasts than the CM of the control vector cells (Fig. 4A and B). We also observed the osteoclastogenesis from osteoclast precursor cell line RAW264.7 cultured with the CM of ITGBL1 KD cells compared with those cultured with CM from control cells (Fig. 4C and D). The TRAP+ osteoclasts induced by CM from ITGBL1 KD cells were significantly smaller and contained fewer nuclei than those induced by the CM from control cells (Fig. 4E and F). Conversely, CM from ITGBL1-overexpressing cells induced more TRAP+ osteoclasts with larger sizes and more nuclei than the CM of the control vector cells (Fig. 4C–F). Taken together, these results suggest that cancer cells with high ITGBL1 expression promoted osteoclast formation and differentiation. Next, we sought to identify osteoclast-activating cytokines affected by ITGBL1 in breast cancer using a human cytokine antibody array and gene expression profile microarray. The mRNA expression levels of interleukins and the secreted protein levels of granulocyte-macrophage colony stimulating factor (GM-CSF), growth-regulated alpha protein (GRO), IL10, monocyte chemotactic protein (MCP) 1, MCP-3, and macrophage stimulating protein alpha (MSP-a) were decreased in the CM of ITGBL1 KD cells compared with the CM of control cells (Supplementary Table S2 and Fig. 4G). The expression levels of RANKL, a pivotal activator of osteoclastogenesis, decreased in the ITGBL1 KD cells and...
Figure 3.
ITGBL1 promotes the attraction of breast cancer cells to osteoblasts and their survival in osteoblast CM. Two ITGBL1 knockdown MDA-MB-231 subclones (ITGBL1 KD1 and ITGBL1 KD2) and two ITGBL1-overexpressing MDA-MB-231 subclones (ITGBL1-1 and ITGBL1-2) were established by stably transfecting MDA-MB-231 cells with shITGBL1 and pEGFP-ITGBL1 plasmids; T47D cells were transfected with pEGFP-ITGBL1 plasmid (ITGBL1).

A and B, Transwell assay for chemotactic migration of cancer cells toward osteoblast-like MG-63 cells (A) or lung epithelial BEAS-2B cells (B). C and D, soft agar colony formation assay of cancer cells in MG-63 CM (C) or BEAS-2B CM (D). E and F, growth curves of cancer cells in MG-63 CM (E) or BEAS-2B CM (F). G, ITGBL1 and proteins related to bone metastasis were detected by Western blot analysis. These assays were performed in triplicate; *, P < 0.05; **, P < 0.01; ***, P < 0.001.
increased in the ITGBL1-overexpressing cells. In contrast, the expression levels of osteoprotegerin, a key inhibitor of osteoclastogenesis, increased in the ITGBL1 KD cells and decreased in the ITGBL1-overexpressing subclones (Fig. 4H). These findings further indicated that breast cancer cells with high ITGBL1 levels, after spreading and surviving in the bone microenvironment, could stimulate osteoclastogenesis, and thereby contribute to osteolytic lesions.
TGFβ mediates the function of ITGBL1 in bone metastasis of breast cancer

Considering the positive correlation between the expression levels of ITGBL1 and TGFβ3 (Fig. 1A) and the downregulation of six genes encoding TGFβ signaling pathway molecules after ITGBL1 KD (Supplementary Table S2), we speculated that TGFβ signaling mediated the ITGBL1-induced promotion of breast cancer bone metastasis. Indeed, we observed increased secretion of TGFβ1 and TGFβ3 by ITGBL1-overexpressing cells (Fig. 5A). Further immunofluorescent staining showed that Smad2 translocated into the nuclei of ITGBL1-overexpressing cells (Fig. 5B), and a Smad2 luciferase reporter assay confirmed that Smad2 transcriptional activity was elevated by ITGBL1 overexpression (Fig. 5C), suggesting that the TGFβ/Smad signaling pathway was activated in ITGBL1-overexpressing cells. Then, we used the TGFβ receptor inhibitor SB-431542 to block the TGFβ/Smad signaling pathway (Fig. 5C). Consistent with TGFβ/Smad signaling pathway activity, the chemotactic responses of MDA-MB-231 cells toward MG-63 cells (Fig. 5D) and the stimulation of osteoclastogenesis (Fig. 5E) were increased by ITGBL1 overexpression and inhibited by SB-431542 treatment. Molecularly, the bone metastasis–related proteins CDH11, CD44, and RANKL and mRNA transcripts of CDH11, OPN, OSN were upregulated in ITGBL1-overexpressed cells, and these changes were reversed by blocking the TGFβ signaling pathway (Fig. 5F, Supplementary Fig. S3A and S3B). These results suggest that the TGFβ signaling pathway mediates the role of ITGBL1 in breast cancer bone metastasis.

**ITGBL1 is a transcriptional target of Runx2**

Because Runx2 is a key transcription factor during bone development and ITGBL1 is coexpressed with RUNX2 and Runx2-regulated bone remodeling-related genes in primary breast cancer tissues, we investigated whether ITGBL1 was regulated by Runx2. Indeed, through a sequence search of the ITGBL1 promoter, we found three OSE2 elements in the proximal promoter region of ITGBL1: −793 to −786, −1208 to −1201 and −1320 to −1295 (Fig. 6A). We performed a ChIP assay to enrich the Runx2-bound DNA fragments and found that Runx2 bound the ITGBL1 promoter region containing the −793 to −786 site but not the −1320 to −1295 or −1208 to −1201 site (Fig. 6B).

We further investigated the regulatory activity of Runx2 on the ITGBL1 promoter using dual-luciferase reporter assays and found that ITGBL1 promoter activity decreased in the MDA-MB-231 cells

**Figure 5.** Blockade of TGFβ signaling reduces the function of ITGBL1 in promoting the chemotactic migration of cancer cells toward osteoblasts and osteoclastogenesis. To block the TGFβ pathway, cells were treated with 1.0 μmol/L SB431542; DMSO was used in the control cells. A, the levels of secreted TGFβ1, TGFβ2, and TGFβ3 in the CM of ITGBL1-overexpressing cells and the vector control cells were detected by dot blot. B, immunofluorescent staining showing the subcellular localization of Smad2 in the above cells. C, the activity of the TGFβ–Smad pathway was evaluated by transiently transfecting a Smad luciferase reporter into the above cells. D, chemotactic migration assay of cancer cells toward MG-63 cells. E, osteoclastogenesis derived from RAW 264.7 cells was detected by TRAP staining. F, the expression of bone metastasis–related molecules was detected by Western blot analysis. OPN, osteoprotegerin. Data, means ± SD in six fields of two independent experiments; *P < 0.05; **P < 0.01; ***P < 0.001 compared with the control cells; # # #P < 0.001 compared with the ITGBL1-overexpressing cells.
transfected with pGL3-ITGBL1 −740/+36 and in cells transfected with pGL3-ITGBL1 −740/+36 with a mutated Runx2-binding motif compared with the control cells transfected with pGL3-ITGBL1 −740/+36 with the wild-type Runx2-binding motif (Fig. 6C). Moreover, we also performed dual-luciferase reporter assays to compare the ITGBL1 promoter activity upon the transient transfection of pGL3-ITGBL1/C0 825/+36 in a stable subclone of MDA-MB-231 with that in shRNA-mediated Runx2 KD, Runx2-overexpressing cells, and their control cells. Runx2 KD weakened and Runx2 overexpression enhanced the activity of the ITGBL1 promoter compared with their respective control cells (Fig. 6D). Together, these results demonstrate that Runx2 regulates the activity of the ITGBL1 promoter by binding to the OSE2 element at −793 to −786.

To further confirm the regulatory effect of Runx2 on ITGBL1 transcription levels, the mRNA expression levels of ITGBL1 in Runx2 KD cells, Runx2-overexpressing cells, and their corresponding control cells were measured by RT-qPCR. The ITGBL1 mRNA expression level was downregulated in Runx2 KD cells and upregulated in Runx2-overexpressing cells compared with their respective control cells (Fig. 6E). These results confirm that ITGBL1 is a positively regulated transcriptional target of Runx2.

ITGBL1 mediates Runx2-driven bone metastasis of breast cancer cells

Once ITGBL1 was confirmed as a Runx2 target gene that plays pivotal roles in breast cancer bone metastasis, we further investigated whether ITGBL1 mediates the Runx2-driven bone metastasis of breast cancer cells. The chemotactic migration assay showed that Runx2 overexpression significantly increased the chemotactic migration of MDA-MB-231 cells toward MG-63 cells. ITGBL1 KD in Runx2-overexpressing cells reversed the Runx2-induced chemotactic migration phenotype (Fig. 7A). We also observed that the chemotactic migration of MDA-MB-231 cells toward MG-63 cells was significantly reduced by Runx2 KD and was rescued by the accompanying ITGBL1 overexpression (Fig. 7A). The culture of preosteoclast RAW 264.7 cells with CM from Runx2-overexpressing cells induced more and larger TRAP⁺ osteoclasts with more nuclei than did control medium (Fig. 7B). Moreover, osteoclast differentiation induced by the Runx2-overexpressing cells was attenuated by ITGBL1 KD (Fig. 7B). Consistently, the CM of Runx2 KD cancer cells reduced osteoclast differentiation in the primary osteoclast culture system, and ITGBL1 overexpression recovered the capability of Runx2 KD cancer cells to activate osteoclasts (Fig. 7C). These phenotypes
ITGBL1 mediates Runx2-driven bone metastasis. MDA-MB-231 cells were transfected with the pcDNA vector combined with pSilencer-control (Control), pcDNA-RUNX2 mixed with pSilencer-control (Runx2 + Control KD), pcDNA-RUNX2 and pSilencer-ITGBL1 (Runx2 + ITGBL1 KD), pSilencer-Runx2 with pcDNA vector (Runx2 KD + vector), and pSilencer-Runx2 combined with pcDNA-ITGBL1 (Runx2 KD + ITGBL1). A, chemotactic migration assay of cancer cells toward MG-63 cells. B, osteoclastogenesis derived from RAW 264.7 cells was detected by TRAP staining. C, osteoclastogenesis derived from primary preosteoclasts was detected by TRAP staining. D, markers for bone metastasis were detected by Western blot analysis. Data, means ± SD in six fields of two independent experiments; *, *P < 0.05; **, *P < 0.01; ***, **P < 0.001 compared with the control cells; #, *P < 0.05; ##, **P < 0.01; ###, ***P < 0.001 compared with the Runx2-overexpressing/Runx2 KD cells.

Discussion
The present study provides clinical and experimental evidence for a role of ITGBL1 in conferring a bone-specific metastasis capability to breast cancer cells. ITGBL1 is highly expressed in bone metastatic tumor cells, as well as in primary cancer cells with bone metastasis capabilities. ITGBL1 facilitates the acquisition of tumor cell advantages in recruiting, residing, and growth in bone and further stimulates osteoclast maturation in the bone microenvironment to form bone metastatic lesions. We also identified a fundamental mechanism of ITGBL1 in bone metastasis via TGFβ pathway activation. Moreover, ITGBL1, as a transcriptional target of Runx2, mediates the role of Runx2 in promoting breast cancer bone metastasis.

Tropism and adhesion are crucial steps in the communication and interaction between breast cancer cells and the bone matrix or host cells in bone. Both clinical data and animal experiments demonstrated the role of ITGBL1 in organ selectivity to the bone during breast cancer metastasis. Furthermore, our evidence that ITGBL1 regulates the expression of chemokine receptor 4 (CXCR4), integrin αv, and CD44 on the surface of breast cancer cells supports the function of ITGBL1 in promoting the bone tropism of breast cancer cells. The chemokine (C-X-C motif) ligand 12 (CXCL12/SDF-1α) and CXCR4 axis is a well-known chemotactic mechanism for breast cancer cell metastasis to bone (21, 25). CXCL12 is expressed at high levels by osteoblasts and bone marrow stromal cells and recruits CXCR4-positive cancer cells to bone marrow (26). Integrin αv on the surface of bone metastatic breast cancer cells binds to RGD domain-containing bone matrix proteins such as OPN, BSP, COLs, thus leading to breast cancer cell adhesion to bone (27–29). CD44, a transmembrane glycoprotein, is a major adhesion molecule for the extracellular matrix (ECM) that binds primarily to the extracellular glycosaminoglycan hyaluronan (HA). HA is a major non-protein glycosaminoglycan component of the ECM in human bone matrix, and the interaction of CD44 and HA facilitates tumor cell arrest and colonization in bone, leading to increased bone metastasis (24, 30).

Metastatic cancer cells that home and localize to the bone marrow can remain dormant (31, 32) until they are activated to colonize and grow into visible metastatic bone lesions. In this study, we found that ITGBL1 was highly expressed in clinical bone metastatic tumors and that ITGBL1 enhanced the colony formation and proliferation capability of cancer cells in a bone-mimicking microenvironment, emphasizing its roles in the potential reprogramming to activate quiescent cancer cells and survival advantage in bone microenvironment. One explanation for this survival advantage is the osteomimetic aspect of cancer cells. Osteomimetic properties of breast cancer cells include the ectopic expression of bone matrix proteins and osteoblast-specific adhesion molecules, and even mineralization under appropriate
conditions (16, 17). These osteoblast-like features allow cancer cells to reside in bone due to their escape from immune surveillance in the bone microenvironment (6, 16, 17). Combining the report that ITGBL1 was identified as a gene in the osteoblast-like gene-expression signature (6) with our finding that ITGBL1 increased the expression of osteomimetic markers, we propose that ITGBL1 contributes to osteomimetic breast cancer, and therefore facilitates cancer cells preferentially residing in and colonizing the bone microenvironment. Moreover, ITGBL1 positively regulated the cell proliferation-related genes KIF6 and CCNE, suggesting that ITGBL1 can also confer a proliferation advantage to tumor cells in the bone microenvironment.

Most breast cancer metastases in bone cause osteolytic lesions. Continuous expansion of osteolytic bone metastasis is driven by the "vicious cycle" of tumor-dependent activation of bone-degrading osteoclasts and bone stroma-dependent stimulation of tumor malignancy (22, 31). Osteoclasts are derived from precursors in the mononuclear-phagocyte lineage and are responsible for bone resorption. RANKL, primarily produced by osteoblasts and cancer cells, binds to its cognate receptor RANK on osteoclast precursors and plays a critical role in promoting osteoclast differentiation and activation, leading to bone resorption (33). Osteoprotegerin is a soluble decoy receptor for RANKL that blocks osteoclast formation by inhibiting the binding of RANKL to RANK (33). Indeed, we observed that high ITGBL1 expression in cancer cells promoted osteoclast activation in vitro and osteolytic lesions in vitro. Consistent with that phenotype, ITGBL1-overexpressing cancer cells showed elevated levels of RANKL and decreased expression of osteoprotegerin. Moreover, ITGBL1 positively regulated the secretion of the osteoclast differentiation activators GM-CSF (34), GRO (35), IL1α (36), MCPs (37), and MIP-α (38) in cancer cells, providing additional evidence supporting the role of ITGBL1 in cancer cell–triggered osteolytic lesions in bone metastatic events.

The role of the TGFβ signaling pathway in bone metastasis has been clearly outlined. TGFβs promote the formation of osteomimetic breast cancer by inducing the expression of bone matrix proteins (17) and also contribute to the "vicious cycle" and osteoclastic resorption (22). In this study, we found that ITGBL1 increased the expression and secretion of TGFβ1 and TGFβ3 in breast cancer cells. Blockade of the TGFβ pathway by SB431542 weakened the ITGBL1-induced bone metastatic potential of breast cancer cells, suggesting a modulatory role for the TGFβ pathway in ITGBL1-driven bone-specific breast cancer metastasis. However, the detailed mechanism of TGFβ upregulation and TGFβ pathway activation by ITGBL1 need to be further explored in future studies.

Rum2 is regarded as a pivotal transcription factor for the formation of the osteomimetic phenotype (39, 40) and contributes to cancer cell dissemination into the blood, survival in the bone microenvironment, and stimulation of bone resorption (41, 42). Expression of Rum2 target genes, including OPN, BSP, and osteocalcin, appears to confer osteomimetic features upon breast cancer cells, and is reported to play important roles in breast cancer bone metastasis (39). In this study, we identified ITGBL1 as a novel Rum2 target gene and demonstrated that ITGBL1 exhibits properties similar to Rum2 in promoting the formation of osteomimetic breast cancer and mediates the Rum2-induced conferral of bone metastasis capability on cancer cells.

In conclusion, our study revealed a new molecular mechanism that promotes breast cancer metastasis to the bone. We also identified an activator of the TGFβ signaling pathway and a key mediator of Rum2 in breast metastasis. Importantly, we provided preclinical evidence for ITGBL1 as a molecular marker to predict bone metastasis risk and as a therapeutic target against breast cancer bone metastasis.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: X-Q. Li, Y-M. Feng
Development of methodology: X-Q. Li, D-M. Li
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): X-Q. Li, D-M. Li, P-Z. Kong, Y. Sun, P-F. Liu, Q-S. Wang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X-Q. Li, X. Du, Y. Sun, P-F. Liu
Writing, review, and/or revision of the manuscript: X-Q. Li, Y-M. Feng
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): P-Z. Kong, Y-M. Feng
Study supervision: Y-M. Feng

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References
1. Sethi N, Dai X, Winter CG, Kang Y. Tumor-derived JAGGED1 promotes osteomimetic breast cancer by inducing the expression of EGF-like motif proteins (22, 33). Osteoprotegerin is a soluble decoy receptor for RANKL that blocks osteoclast formation by inhibiting the binding of RANKL to RANK (33). Indeed, we observed that high ITGBL1 expression in cancer cells promoted osteoclast activation in vitro and osteolytic lesions in vitro. Consistent with that phenotype, ITGBL1-overexpressing cancer cells showed elevated levels of RANKL and decreased expression of osteoprotegerin. Moreover, ITGBL1 positively regulated the secretion of the osteoclast differentiation activators GM-CSF (34), GRO (35), IL1α (36), MCPs (37), and MIP-α (38) in cancer cells, providing additional evidence supporting the role of ITGBL1 in cancer cell–triggered osteolytic lesions in bone metastatic events.

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ITGBL1 in Breast Cancer Bone Metastasis

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Xiao-Qing Li, Xin Du, Dong-Mei Li, et al.


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