

Human Bone Marrow–Derived MSCs Can Home to Orthotopic Breast Cancer Tumors and Promote Bone Metastasis

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Abstract

American women have a nearly 25% lifetime risk of developing breast cancer, with 20% to 40% of these patients developing life-threatening metastases. More than 70% of patients presenting with metastases have skeletal involvement, which signals progression to an incurable stage. Tumor–stroma cell interactions are only superficially understood, specifically regarding the ability of stromal cells to affect metastasis. *In vivo* models show that exogenously supplied human bone marrow–derived stem cells (hBMSC) migrate to breast cancer tumors, but no reports have shown endogenous hBMSC migration from the bone to primary tumors. Here, we present a model of *in vivo* hBMSC migration from a physiologic human bone environment to human breast tumors. Furthermore, hBMSCs alter tumor growth and bone metastasis frequency. These may home to certain breast tumors based on tumor-derived TGF- β 1. Moreover, at the primary tumor level, interleukin 17B (IL-17B)/IL-17BR signaling may mediate interactions between hBMSCs and breast cancer cells. *Cancer Res*; 70(24); 10044–50. ©2010 AACR.

Introduction

Approximately 200,000 new cases of breast cancer and 40,000 deaths from breast cancer occur annually in the United States, making this the most common malignancy and second leading cause of cancer death in women (1). Metastasis of breast cancer cells (BCC) from the primary neoplasm to the bone marrow can occur in up to 70% of breast cancer cases, which often signals an incurable progression (2). Many mechanisms behind the metastatic process still remain unclear, particularly the roles of supportive tissue, but evidence is building for the central role of stromal cells. Such cells include bone marrow–derived mesenchymal stem cells (BMSC), which are known to home to tumors, likely due to

the tumor's chronic inflammation, and promote metastasis (3–5).

To our knowledge, ours is the first *in vivo* breast cancer model used to study the contribution of BMSCs from a human bone microenvironment (hBMSCs) or the effects of hBMSCs on breast cancer skeletal metastasis (5). Herein, we show that hBMSCs from a human bone environment can home to orthotopically implanted human breast cancer tumors and alter BCC proliferation and the frequency of visceral and skeletal metastases. These novel models describe how BCCs with different tissue tropisms and metastatic frequencies home to, and respond uniquely to, hBMSCs and propose transforming growth factor β (TGF- β) and interleukin 17B (IL-17B) as mediators of these tumor–stroma cell interactions.

Materials and Methods

Cell culture

Cells were isolated and cultured as described previously or as recommended by the ATCC (American Type Culture Collection; refs. 3, 6–9), and hBMSCs were validated for pluripotency (Supplementary Fig. S1). BCCs were obtained from ATCC, donated from the laboratory of Steve Ethier (Karmanos Cancer Institute, Wayne State University, Detroit, MI), or isolated in our laboratory (SUM1315-BP2) and were validated for correct morphology using phase-contrast microscopy throughout the full experiment by comparing with ATCC images or the parental cell line (SUM1315-BP2). BCCs expressed the firefly luciferase reporter gene. hBMSCs were fluorescently labeled using Vybrant DiI or DiD according to manufacturer's directions (Molecular Probes).

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hBMSC differentiation

Osteogenic and adipogenic differentiation media and Oil Red-O and Alizarin Red staining for validation of hBMSC pluripotency have been previously described (9, 10).

Conditioned media, proliferation, and migration assays

Conditioned media (CM) were derived by applying serum-free medium to cells at 80% confluence for 24 hours. Cell proliferation was measured using an MTT-based assay (Roche Diagnostics) according to the manufacturer's instructions. Migration was assessed according to the manufacturer's instructions (Neuroprobe or Cultrex) and fixing, drying, (Diff Stain Kit; IMEB), and counting (ImageJ; NIH) cells, manually or with fluorescence emission. For IL-17B migration assays, 20 ng/mL of rhIL-17B (R&D Systems) was added to BCCs before seeding.

Engineered bone for tumor tropism

Silk scaffolds were created and differentiated into tissue-engineered bone (TEB) as previously reported (9). Specifically, scaffolds seeded with 1×10^6 P2 hBMSCs were differentiated *in vitro* for 2 weeks and reseeded with 1×10^6 fluorescently labeled P3 hBMSCs 1 day before implantation, which were tracked for tumor tropism.

Animal experiments

The Tufts University Division of Laboratory Animal Medicine and Institutional Animal Care and Use Committee approved all animal procedures. Metastasis and hBMSC-tumor homing procedures, including imaging, TEB implantation, tumor formation, and histology were described previously (6, 9). Two weeks after TEB implantation, mice were sacrificed, and tumors and TEB were removed for analysis.

Primary tumor digestion and FACS analysis

Primary tumors were removed and digested as described previously in ref. 11). Fluorescence-activated cell sorting (FACS) was done on a MoFlo Cell Sorter (Beckman-Coulter) and analyzed using FloJo Software v. 7.5.4.

SUM1315-BP2 creation and isolation

SUM1315 BCCs (1×10^6) were injected into human trabecular bone fragments implanted subcutaneously into non-obese diabetic/severe combined immune deficiency (NOD/SCID) mice using a 22-gauge needle and Hamilton syringe (Supplementary Fig. S2). Fragments were harvested at 3 to 4 months, BCCs were isolated and 1×10^6 "SUM1315-BP1" cells were again directly injected into subcutaneous bone implants, which were harvested at 3 to 4 months for SUM1315-BP2 BCC line isolation.

RNA isolation and quantitative RT-PCR

Total RNA was extracted in TRIzol reagent (Invitrogen), and quantitative RT-PCR was performed according to manufacturer's instructions (Bio-Rad). Primers were designed using PrimerBank (<http://pga.mgh.harvard.edu/primerbank/>).

il-17br overexpression

A plasmid containing the IL-17BR cDNA was obtained from PlasmID (<http://plasmid.med.harvard.edu/PLASMID/>). cDNA was cloned into pLenti6.2/V5-DEST (Invitrogen), used to create lentiviral particles and infect BCCs according to the manufacturer's directions.

Statistics

Data are represented as mean \pm SEM, and significance was determined using a 2-sided Student's *t* test, unless otherwise noted (**P* < 0.05; ***P* < 0.01; *** *P* < 0.001).

Results and Discussion

The SUM1315 human BCC line is a highly aggressive, particularly osteotropic (6, 9) cell line derived from a metastatic nodule from a patient with invasive ductal carcinoma. By passaging SUM1315 BCCs through human bone, we created the SUM1315-BP2 cell line with a unique gene expression signature that represents a "bone-educated" cell line (Supplementary Fig. S2). The MDA-MB-231 and MCF7 BCC lines represent a widely metastatic and noninvasive cell line.

Similar to previous reports (3), hBMSCs migrated toward BCC-conditioned media (BCC-CM) *in vitro* with an increased affinity for highly aggressive and bone-metastatic BCCs (MDA-MB-231 and SUM1315) as compared with less aggressive BCCs (MCF7; Supplementary Fig. S3). To analyze the ability of hBMSCs to home from the bone environment to mammary fat pad (MFP) tumors *in vivo*, we incorporated previously developed TEB into a novel hBMSC-tumor homing model (9). In the model, TEB delivers fluorescently labeled hBMSCs, which migrate to breast cancer tumors as assessed by confocal imaging and FACS (Supplementary Fig. S4; Fig. 1A). Similar to *in vitro* results, SUM1315 and MDA-MB-231 could statistically attract hBMSCs *in vivo*. No significant difference from background signals was observed for the MCF7 and SUM1315-BP2 tumors (Fig. 1B). Implanted TEB retained a bone phenotype and fluorescent hBMSCs for the duration of the experiment (2.5 weeks; Supplementary Fig. S5).

Clinically, TGF- β 1 is elevated in the plasma of breast cancer patients and is linked to increased cancer progression and metastasis (12). In addition, TGF- β 1 is produced by aggressive BCCs and can attract hBMSCs in *in vitro* assays and physiologic bone development (refs. 11, 13; Supplementary Fig. S6). We hypothesized that hBMSCs migrate toward BCCs in response to elevated TGF- β 1 levels and confirmed this *in vitro* (Supplementary Fig. S6). In addition, blockade of TGF- β 1 in BCC-CM by using a neutralizing antibody significantly reduced hBMSC migration (Supplementary Fig. S6). TGF- β 1 blockade did not inhibit hBMSC migration toward MDA-MB-231-CM, perhaps due to the abundance of other cytokines in the supernatant. These results further support the theory that inflammation attracts hBMSCs to tumors and suggest that TGF- β 1 may play a large role in attracting hBMSCs.

Consistent with published reports, hBMSCs showed tumor-type dependent effects on BCC proliferation *in vitro* and *in vivo* (refs. 3, 5; Fig. 2; Supplementary Fig. S7). When cultured with

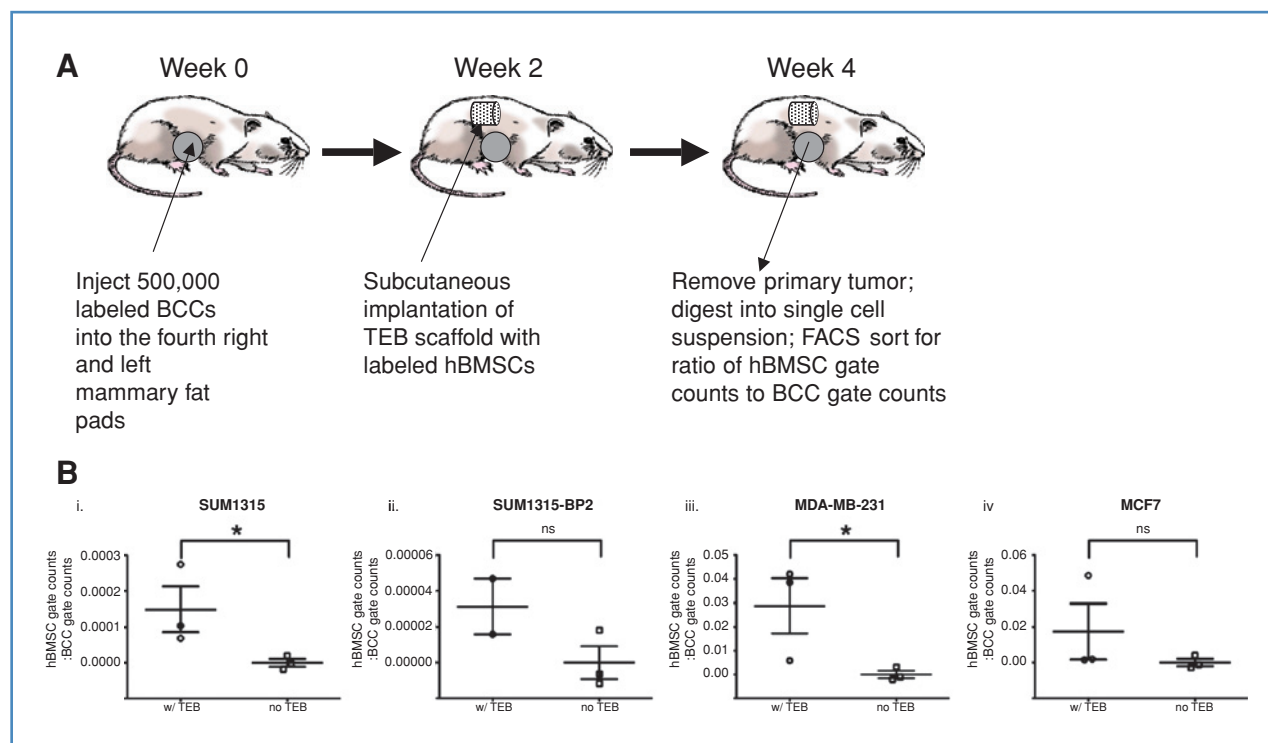


Figure 1. hBMSCs from the human bone environment migrate to orthotopic BCC tumors *in vivo*. A, schematic of the *in vivo* model of hBMSC migration from the bone environment to primary BCC tumors. B, FACS analysis of tumors explanted and digested from mice. The ratio of counts within both gates (hBMSC and BCC gates) for tumors from mice implanted with ("w/TEB") and without TEB ("no TEB") was compared for each tumor type. No TEB mice were implanted with unseeded scaffolds. Mice were analyzed with primary tumors of BCCs of SUM1315 (i), SUM1315-BP2 (ii), MDA-MB-231 (iii), and MCF7 (iv). Gating of fluorescence from hBMSCs and BCCs was based on labeled (positive) and unlabeled (negative) single-cell controls. The y-axis (hBMSC gate counts:BCC gate counts) represents the ratio of counts within the fluorescent gates for each tumor type. Statistics utilized a 1-sided Student's *t* test. *, $P < 0.05$; ns, $P > 0.05$.

CM from bone-derived cell types, SUM1315 cells showed increased proliferation. In contrast, MDA-MB-231 cells showed no proliferative response (Supplementary Fig. S7). The weakly metastatic MCF7 cell line shows yet a third response to hBMSC-CM, that is, decreased proliferation (Supplementary Fig. S7). Following 72 hours of coculture with hBMSC-CM, no morphologic changes were observed in the 3 BCC types analyzed, although we cannot rule out that longer culture may promote epithelial-to-mesenchymal morphologic changes as observed previously ref. 14; data not shown). We next utilized orthotopic (MFP) implantation to assess *in vivo* proliferative changes of BCCs with or without hBMSCs, as local tumor microenvironment strongly influences cancer growth and progression (15). Consistent with our *in vitro* findings, SUM1315 BCCs coinjected with hBMSCs in a ratio consistent with previous reports (ref. 3; BCCs:hBMSCs = 1:3) showed increased tumor growth compared with BCCs injected alone whereas no effect or a negative effect was seen for MDA-MB-231 and MCF7 tumors, respectively (Fig. 2). When cocultured with hBMSCs, SUM1315-BP2 BCCs show similar proliferative changes to parental SUM1315 BCCs *in vitro* (data not shown) and *in vivo* (Fig. 2). These *in vitro* and *in vivo* data suggest a unique response of BCCs to hBMSCs depending on their metastatic propensity and tissue tropisms.

Because tumor growth does not necessarily correlate with metastatic outcome (3), we next investigated whether hBMSCs cocultured with BCCs could increase BCC migration *in vitro* or metastasis *in vivo*. Similar to previously published data, we observed that indirect coculture of hBMSCs with MDA-MB-231 BCCs resulted in increased migration of BCCs *in vitro* (Fig. 3A; ref. 16). Conversely, hBMSC coculture decreased migration of SUM1315 BCCs in a Transwell migration assay (Fig. 3A). Interestingly, hBMSC-CM stimulation increased SUM1315-BP2 migration, suggesting that bone preconditioning adapts BCCs to utilize bone-derived signals to increase their migration. No significant *in vitro* migration was observed for MCF7 BCCs with or without indirect hBMSC coculture (data not shown). To assess metastasis after 10 weeks *in vivo*, specifically to bone, we utilized a humanized model of breast cancer metastasis to bone developed in our laboratory (6, 9). Coinjection of MDA-MB-231 BCCs and hBMSCs into the MFP increased the frequency of metastasis to human bone, lung, and liver (Fig. 3B). Similar to our *in vitro* results, no increase in metastasis frequency was observed for SUM1315 BCCs (Fig. 3B). SUM1315-BP2 BCCs coinjected with hBMSCs exhibited an increased skeletal metastasis frequency (Fig. 3), mimicking effects found *in vitro*.

The gene expression signature of SUM1315-BP2 BCCs is enriched for genes that may promote metastasis (Supplementary

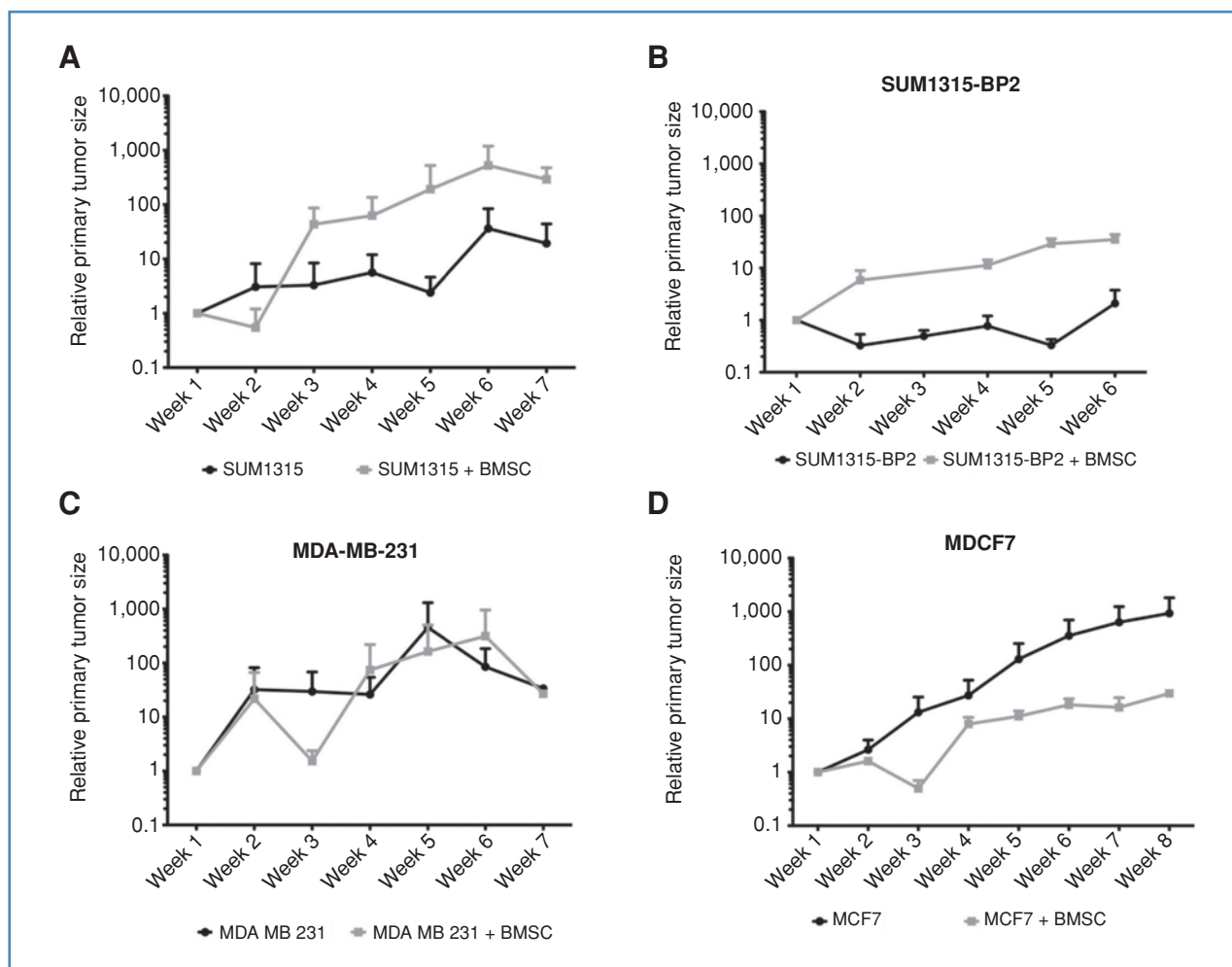


Figure 2. hBMSCs affect BCC growth *in vivo*. 500,000 SUM1315 (A), SUM1315-BP2 (B), MDA-MB-231 (C), and MCF7 (D) BCCs were injected alone or with 1.5×10^6 hBMSCs into the MFP. Tumor volume was measured with bioluminescent imaging weekly. Data are normalized to bioluminescence from the first week of measurement and represented as mean \pm SEM ($n = 5$ for all groups). Statistics performed using 2-way ANOVA or 2-sided Student's *t* test on endpoint: SUM1315, *, $P = 0.0122$; SUM1315-BP2, ***, $P = 0.0001$; MDA-MB-231, ns, $P = 0.9282$; MCF7, ns, $P = 0.0875$.

Table S1), 4 of which were confirmed to be overexpressed using quantitative RT-PCR (refs. 17, 18; Supplementary Fig. S2). One of these genes, IL-17b receptor (*il-17br*), is a prognostic indicator of breast cancer progression and metastasis and, along with its ligand, IL-17b (*il-17b*), is linked to bone turnover and tumor progression (17, 19, 20). In addition, hBMSCs secrete IL-17B, which may stimulate metastasis through IL-17BR (21). To see whether *il-17br* was consistently overexpressed in skeletal metastases, we utilized our *in vivo* bone metastasis model to assess gene expression levels of skeletal metastases from 3 metastatic BCC lines (SUM1315, SUM1315-BP2, and MDA-MB-231). MCF7 BCCs do not metastasize to the human bone fragment in our *in vivo* bone metastasis model and thus could not be used in this analysis. All 3 metastatic BCC lines metastasized to human bone fragments and mouse hind limbs (Supplementary Fig. S8). Quantitative RT-PCR analysis of the result-

ing metastases showed that *il-17br* was overexpressed in most metastases to human bone cores when compared with their matched primary tumors and was overexpressed greater than 30-fold in bone core metastases from the SUM1315-BP2 BCC samples (Fig. 4A). The lack of overexpression in MDA-MB-231 bone metastases may represent that this highly invasive BCC line does not rely as heavily on IL-17BR for metastasis. We hypothesized that hBMSC-derived IL-17B may stimulate migration of metastatic BCCs. Indeed, stimulation of BCCs with IL-17B resulted in increased migration of the metastatic SUM1315, SUM1315-BP2, and MDA-MB-231 cells *in vitro*, with no observed increase in migration of nonmetastatic MCF7 BCCs (Fig. 4A). In addition, lentiviral overexpression of *il-17br* in MDA-MB-231 and SUM1315 BCCs increased migration *in vitro* and increased frequency of lung and liver metastases *in vivo* (Supplementary Fig. S9; Fig. 4C and D).

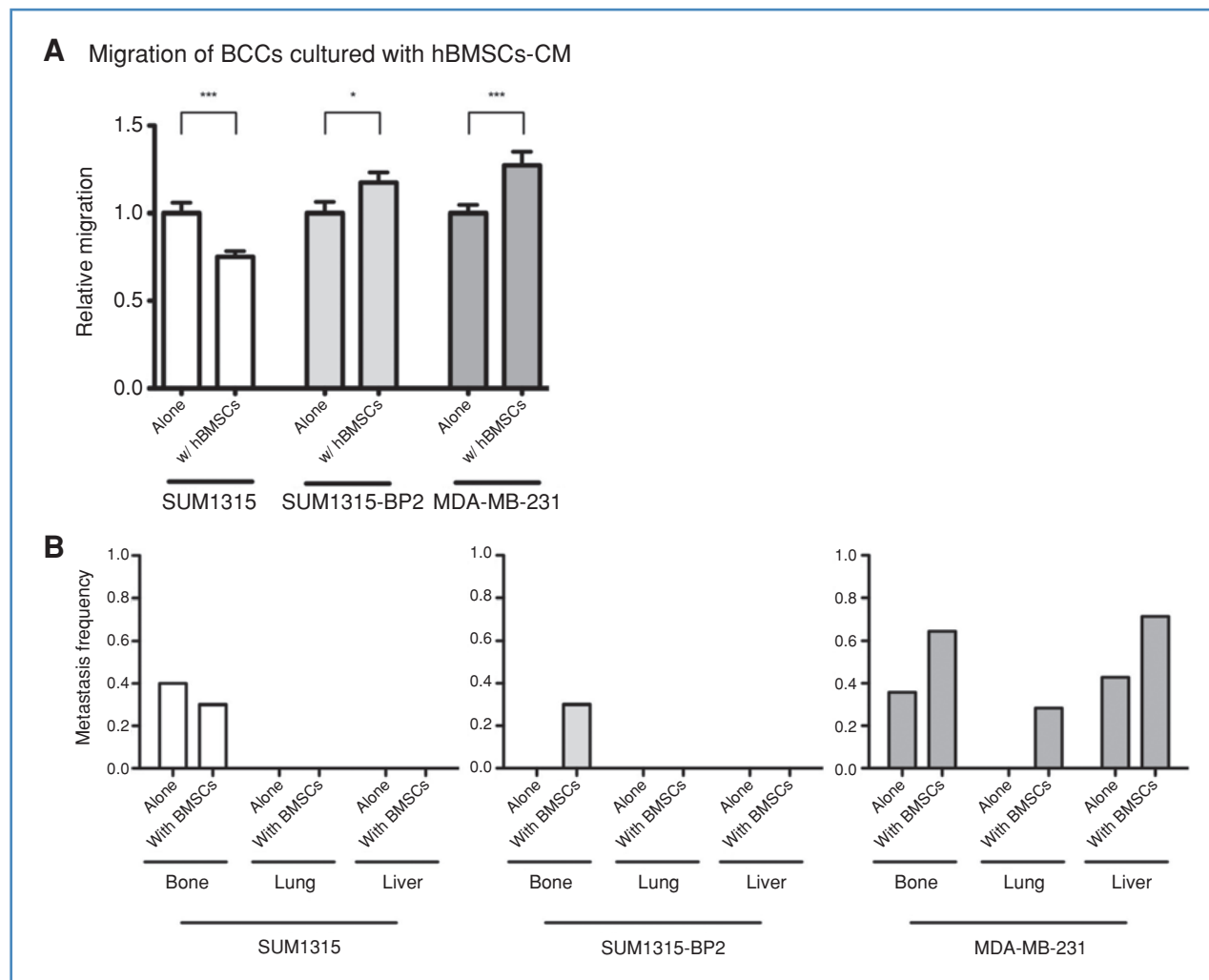


Figure 3. hBMSCs affect migration and metastasis of BCCs. A, SUM1315, SUM1315-BP2, and MDA-MB-231 BCCs were grown in hBMSC-CM for 24 hours. BCCs migrated for 6 hours on Transwell migration assays. Values represent average number of cells, normalized to cancer cell samples without hBMSCs for each BCC line. B, 500,000 BCCs were injected alone or with 1.5×10^6 hBMSCs into the MFP of 10-week-old NOD/SCID female mice harboring implanted human bone cores. After 10 weeks, mice were sacrificed and human bone cores, lungs, and livers were analyzed for metastases by bioluminescence. Metastases were counted, and frequency was calculated by dividing number of metastatic samples by total number of samples ($n = 6$; SUM1315 and SUM1315-BP2 with hBMSC; $n = 5$; SUM1315 and SUM1315-BP2 alone; $n = 7$; MDA-MB-231). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Together, these results suggest a possible mechanism by which recruited hBMSCs may stimulate different BCCs to metastasize; IL-17B secretion from hBMSCs may stimulate migration of BCCs through IL-17BR (Supplementary Fig. S10).

hBMSCs are a pluripotent source of growth and stimulatory factors that have been explored for their ability to home to tumor cells for drug delivery applications (5). However, as shown here, the roles of hBMSCs are diverse and tumor-type dependent and their therapeutic potential should not be exploited until more is understood about their inherent pro- and antitumor properties. Here, we have shown for the first time that human BMSCs from a physiologic bone environment can home to orthotopically implanted primary

human breast tumors. The addition of hBMSCs to the primary tumor environment influences tumor proliferation and metastasis. Although many have observed that different breast carcinomas have different proliferative and metastatic capacities based on genetic differences, our data suggest that these variations may also be a result of different abilities to attract, and be stimulated by, hBMSCs that arrive at the primary tumor environment. In addition, not all primary tumors attract hBMSCs and their effects may not be universal. Further work will confirm whether a blockade of hBMSC migration to, or stimulation of, cancer cells can abrogate breast cancer metastasis. Nonetheless, the results presented earlier are promising and suggest that these "healthy" cells are playing a larger role in cancer metastasis than previously thought.

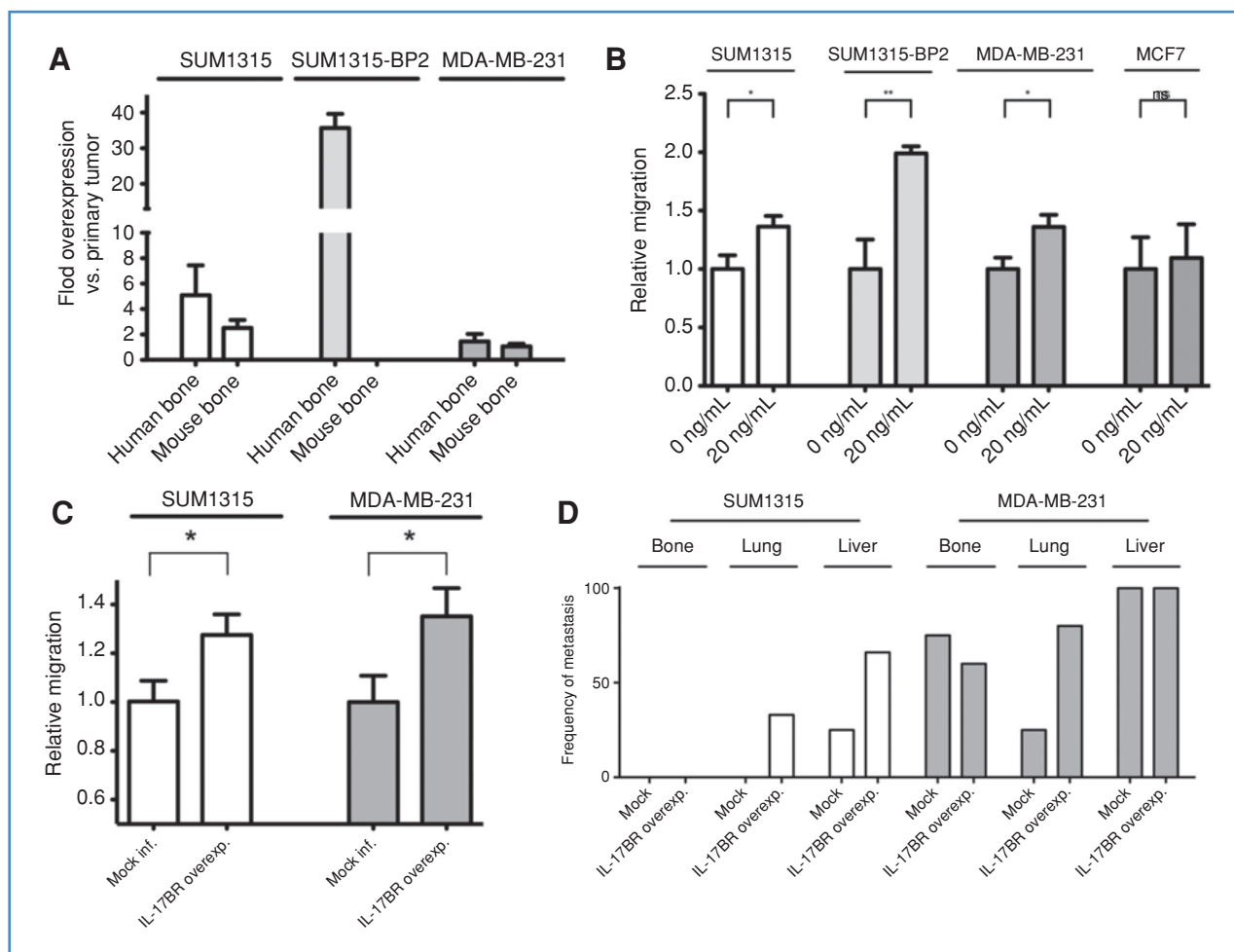


Figure 4. IL-17B stimulation of IL-17BR can drive migration and metastasis. A, expression of *il-17br* was analyzed using quantitative RT-PCR. Metastatic cells were compared with matched primary tumors. $n = 3$ except for the SUM1315-BP2 mouse bone data point where too few metastases inhibited significance. B, serum-starved SUM1315, SUM1315-BP2, and MDA-MB-231 BCCs showed a dose-dependent reaction to IL-17B stimulation after 6 hours. No increase in migration was observed for MCF7 BCCs. C, serum-starved SUM1315 and MDA-MB-231 BCCs (mock infected or infected with lentivirus to overexpress *il-17br* cDNA) migrated for 6 hours on Transwell migration assays. D, bioluminescent imaging of human bone fragments, lungs, and livers was used to assess metastasis frequency. *, $P < 0.05$; ns, $P > 0.05$.

Disclosure of Potential Conflicts of Interest

The authors declare no competing financial interests.

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