Metastatic Breast Cancer Cells Induce Osteocyte Senescence Contributing to Osteolytic Bone Destruction

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ABSTRACT

Breast cancer (BCa) bone metastases cause extensive bone destruction and are typically incurable. We studied the impact of BCa bone metastasis on osteocytes (Ots) - recently identified as key cells of the tumor niche – using single-cell RNA sequencing (scRNAseq). First, we injected murine E0771 BCa cells or PBS into 8-wk female NuTRAP reporter mice crossed with Dmp1-8kb-Cre mice and, after 2 wks, sorted Dmp1-8kb-GFP+ cells to study Ots. Bioinformatic analyses showed that Ots from BCa bone metastases had upregulation of genes enriched in GO terms linked to senescence, senescence-associated secretory phenotype (SASP), and inflammatory response, and had an increased senescence/SASP score compared to naïve mice. Histology revealed 4 times more p16+ (RNAscope) and senescence-associated distension of satellites (SADS)+ (2 vs 14%) Ots, hallmarks of cellular senescence, in the cortical bone of BCa-bearing mice. Injection of human MDA-MB-231 BCa cells into 8-wk female NSG immunodeficient mice also resulted in increased number of telomere-associated DNA-damage foci (TAF), the most specific marker of cellular senescence, in the Ots. Remarkably, iliac crest bone biopsy samples from metastatic breast cancer patients unveiled the close proximity of senescent Ots to BCa cells. Conditioned media (CM) from E0771 or MDA-MB-231 BCa cells increased senescence-associated β-galactosidase activity and upregulated senescence-related genes p16, p21, Mmp13, and Il6 in cultured Ots. Additonally, MDA-MB-231 BCa cells led to similar gene expression changes in ex vivo organ cultures of human bone. Collectively, these results support the idea that signals from metastatic BCa cells cause premature Ot senescence. To assess the impact of Ot senescence on bone mass, we treated 8-wk female mice with intratibial E0771 BCa tumors with a cocktail of the senolytics (D+Q) Dasatinib (5 mg/kg) and Quercetin (50mg/kg) once a wk, starting two days after tumor inoculation. D+Q, known to eliminate senescent cells, prevented the histological increases in p16+ and SADS+ Ots induced by BCa cells. D+Q did not affect tumor burden but decreased lytic lesions and preserved cancellous bone mass (30% higher vs untreated BCa-mice). scRNAseq showed Ots from BCa-bones had elevated Rankl, Mmp13, Il6, Lgals3, Serpine2, Cd9, and Vegfa, and reduced Cthrc1 expression, suggesting increased osteoclastogenic potential. Consistent with this, D+Q restored the elevated p21, p16, and Mmp13 mRNA levels and reduced by 40% the increase in CTX in bones-bearing BCa cells cultured ex vivo. D+Q did not affect P1NP levels, which remained decreased in bones with BCa cells, suggesting senolytics preserved bone by inhibiting resorption. Our data demonstrate that metastatic BCa cells change the bone microenvironment and cause Ot senescence, which in turn induces osteoclastogenesis and osteolytic disease.

BACKGROUND

Bone provides a favorable environment for cancer cells to thrive and is the most common site of invasion of most metastasizing tumors, including breast cancer (BCa). BCa metastases to the bone occur in approximately 70% of patients (1). Bone metastatic BCa causes secondary complications such as osteolytic lesions, pain, and pathological fractures, decreasing quality of life and survival. Once BCa metastasizes to the bone, it is uncurable (2, 3). Thus, a better understanding of the cellular and molecular events that support BCa progression in bone is needed to identify new therapeutic targets to treat this devastating disease.

The bone microenvironment is recognized as a critical player in tumor progression and a promising therapeutic target in cancer. The bone metastatic tumor microenvironment (TME) comprises multiple cells, including osteocytes (Ots), osteoclasts, osteoblasts, adipocytes, endothelial cells, and immune cells. Metastatic BCa cells hijack the bone and establish tumor-host cell interactions via physical cell-to-cell contact or exchange of secreted proteins, creating a TME that supports tumor growth and bone destruction (4). Yet, studies on bone metastatic TME have been limited mainly to the crosstalk between BCa cells and osteoblasts and osteoclasts, known as the vicious cycle of bone metastasis.

Osteocytes comprise ~95% of all bone cells and regulate bone mass throughout life. Although buried within the bone mineral, Ots communicate with other cells via cytoplasmic projections and the distribution of secreted molecules (5). However, the impact of BCa tumors on Ot biology and the contribution of Ots to the development and progression of BCa bone metastasis is not entirely understood.In this study, we demonstrate that metastatic BCa cells induce premature cellular senescence and a distinctive pro-inflammatory senescence-associated secretory phenotype (SASP) in the bone cells, including Ots. Further, we showed that senescent Ots contribute to the aggressive osteolytic disease induced by metastatic BCa cells by promoting osteoclastogenesis.

METHODS

Animal studies. We generated NuTRAP-/+;DMP1-8kb-Cre-/+ reporter mice by crossing B6;129S6-Gt(ROSA)26Sortm2(CAG-NuTRAP)Evdr/J mice (NuTRAP; #029899; Jackson Laboratory, ME, US) with DMP1-8kb-Cre mice. 8-week-old C57BL/6 female mice were injected intratibially with 105 EO771-luc cells or saline and three days later randomized by body weight to the following groups: 1) naïve mice orally receiving vehicle, 2) EO771-luc-bearing mice orally receiving vehicle, or 3) EO771-luc-bearing mice orally receiving a senolytic cocktail (DQ) of Dasatinib (5mg/kg) and Quercetin (50 mg/kg) once a week.

10X Genomics Single-cell RNA sequencing. Cells were isolated from the tibias of NuTRAP-/+; DMP1-8kb-Cre-/+ mice and were encapsulated using a Chromium Controller (10X Genomics, Pleasanton, CA, US), and libraries were constructed using a Chromium Single Cell 3' Reagent Kit (10X Genomics). The libraries were sequenced using an Illumina NovaSeq 600 machine to generate fastq files

Cell cultures. Cell culture studies were performed by treating osteocyte-like cells (OCY454) cultured at 37C for 2 wks with conditioned media (CM) (50%) from breast cancer cells (E0771) for 9 days.

Ex-vivo bone culture. Ex-vivo murine bone cultures were established with femurs from C57BL/6 (EO771 cells) or NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG; #005557, Jackson's Lab) female mice (MDA-MB-231).

RESULTS

Osteocytes from bones with breast cancer (BCa) tumors exhibit upregulation of genes associated with cellular senescence

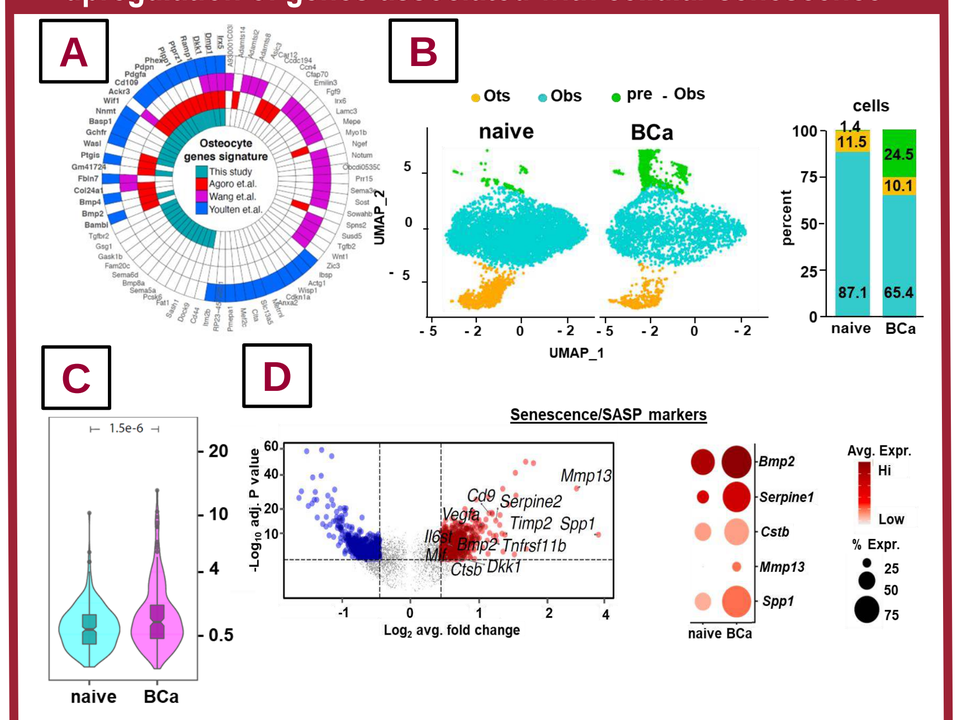

Figure 1. **(A)** Polar plot showing osteocyte (Ot) gene markers identified in our dataset compared to those previously reported by Agoro et al., Wang et al., and Youlten et al. **(B)** Uniform Manifold Approximation and Projection (UMAP) plot representations of osteoblastic cells isolated from control (naïve) or mice with breast cancer bone metastasis (BCa) showing three clusters: Ots, osteoblasts (Obs), and pre-osteoblasts (pre-Obs) and cluster cell distribution by group. **(C)** Comparison of the senescence score in Ots from naïve vs. BCa mice. **(D)** Volcano plot ranking genes according to their relative abundance (log_2 fold change) and statistical value ($-log_{10}$ p-value). Dots show significant upregulated (red) and down-regulated (blue) genes in Ots from naïve vs. BCa mice. Bubble plot comparing expression of selected senescent markers in osteocytes Ots from naïve vs. BCa mice. Bubble size is proportional to the percentage of cells in each cluster expressing a gene, and color intensity is proportional to average scaled gene expression within a cluster.

Metastatic BCa cells induce cellular senescence in osteocyte-like cells

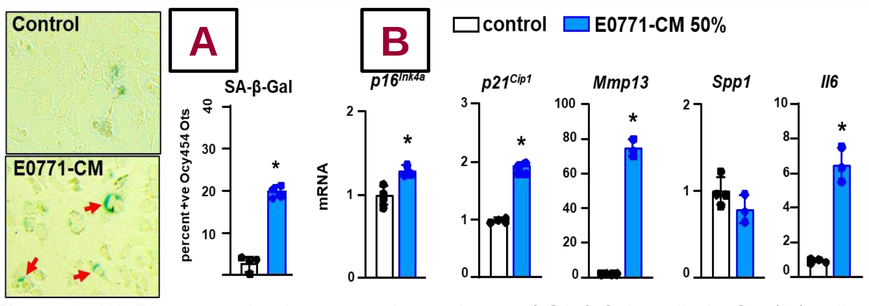

Figure 2. **(A)** Representative images and prevalence of SA-β-Gal+ cells in Ocy454 cells treated with vehicle (control) or conditioned media (CM) from murine EO771 BCa cells for 9 days. **(B)** Expression of senescence markers $p16^{Ink4a}$ and $p21^{Cip1}$ and SASP-related genes Mmp13, Il6, and Spp1. n=3-4/group. *p<0.05 vs. vehicle by t-test. Data are shown as mean ± SD; each dot represents an independent sample; representative experiments out of two are shown.

Metastatic BCa cells increase the expression of senescence markers and SASP factors in primary murine osteocytes

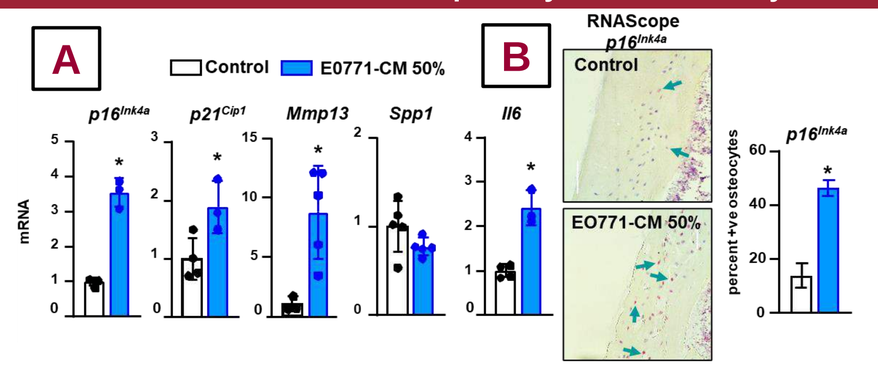

Figure 3. **(A)** Expression of senescence markers $p16^{Ink4a}$ and $p21^{Cip1}$ and SASP-related genes Mmp13, Spp1, and Il6, and **(B)** prevalence of $p16^{Ink4a}$+ primary osteocytes in bones treated with vehicle or conditioned media (CM) from murine EO771 BCa cells cultured ex vivo for five days. n=3-5/group. *p<0.05 vs. vehicle by t-test. Data are shown as mean ± SD; each dot represents an independent sample; representative experiments out of two are shown.

Infiltration of metastatic BCa cells upregulates senescence and SASP-related genes in human bones

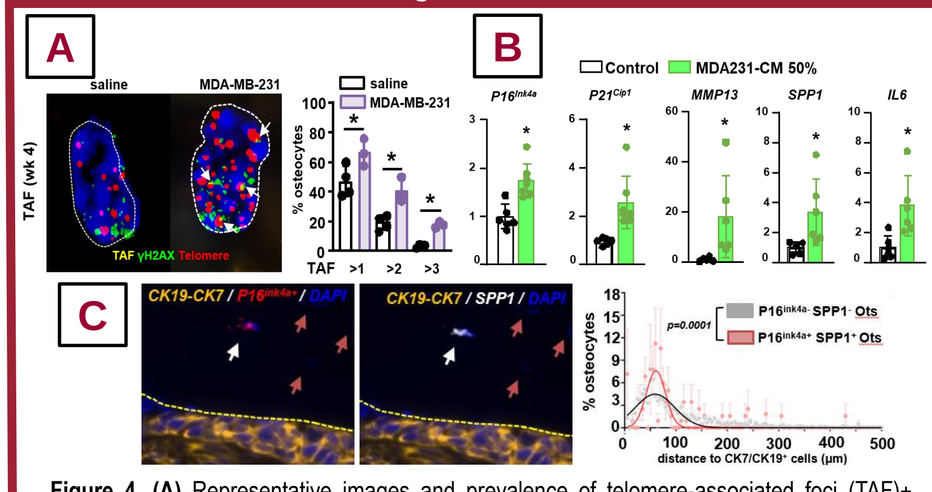

Figure 4. **(A)** Representative images and prevalence of telomere-associated foci (TAF)+ primary osteocytes in bones from naïve and MDA-MB-231 inoculated mice. White arrows indicate TAF events. White dashed lines indicate the nuclei's contour. n=3-4 mice/group. **(B)** Expression of senescence markers and SASP-related genes in human bones treated with vehicle or CM from human MDA-MB-231 breast cancer cells cultured ex vivo for five days. **(C)** Representative image a bone biopsy from a breast cancer patient with bone metastasis stained for CK19-CK7 (orange), P16Ink4a (red), SPP1 (white), and DAPI (blue). White arrows point to $P16^{Ink4a}$+SPP1+ osteocytes, and red arrows point to $P16^{Ink4}$-SPP1- osteocytes. AI-assisted quantitative analysis of the distance to breast cancer cells (CK7/CK19+) distribution for $P16^{Ink4a}$+SPP1+ osteocytes, n=4.

Senolytics deplete senescent Ots in mice with BCa bone metastasis

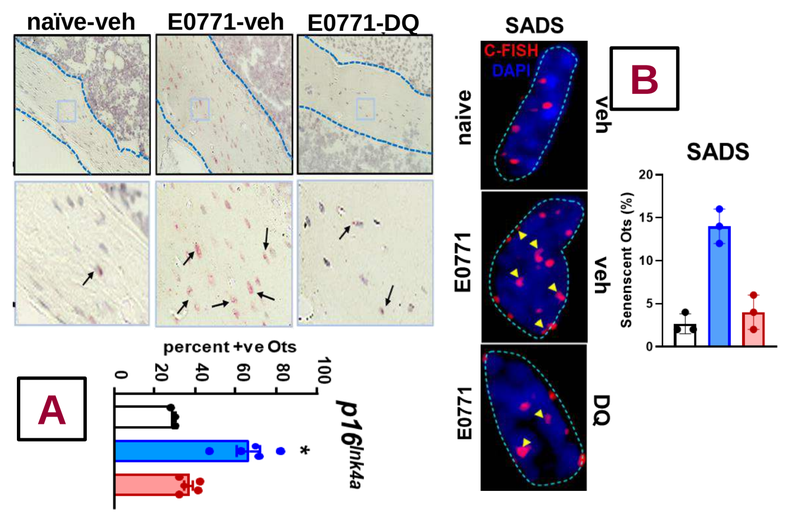

Figure 5. **(A)** Representative images and prevalence of (A) $p16^{Ink4a}$+ (n=6 mice/group; 40X), (B) senescence-associated distension of satellites (SADS)+ (n=3 mice/group; 100X oil), in bones from naïve and BCa mice receiving veh or DQ three weeks after tumor inoculation. Black arrows indicate $p16^{Ink4a}$+ Ots. Blue dashed lines indicate the bone surface. Yellow arrows indicate SADS events. Blue dashed lines indicate the nuclei's contour. C-FISH: centromere-FISH. Yellow dashed lines indicate the bone surface. *p<0.05 vs. vehicle by One Way ANOVA. Data are shown as mean ± SD; each dot represents an independent sample.

Pharmacologic depletion of senescent cells mitigates the osteolytic bone loss induced by BCa bone metastasis.

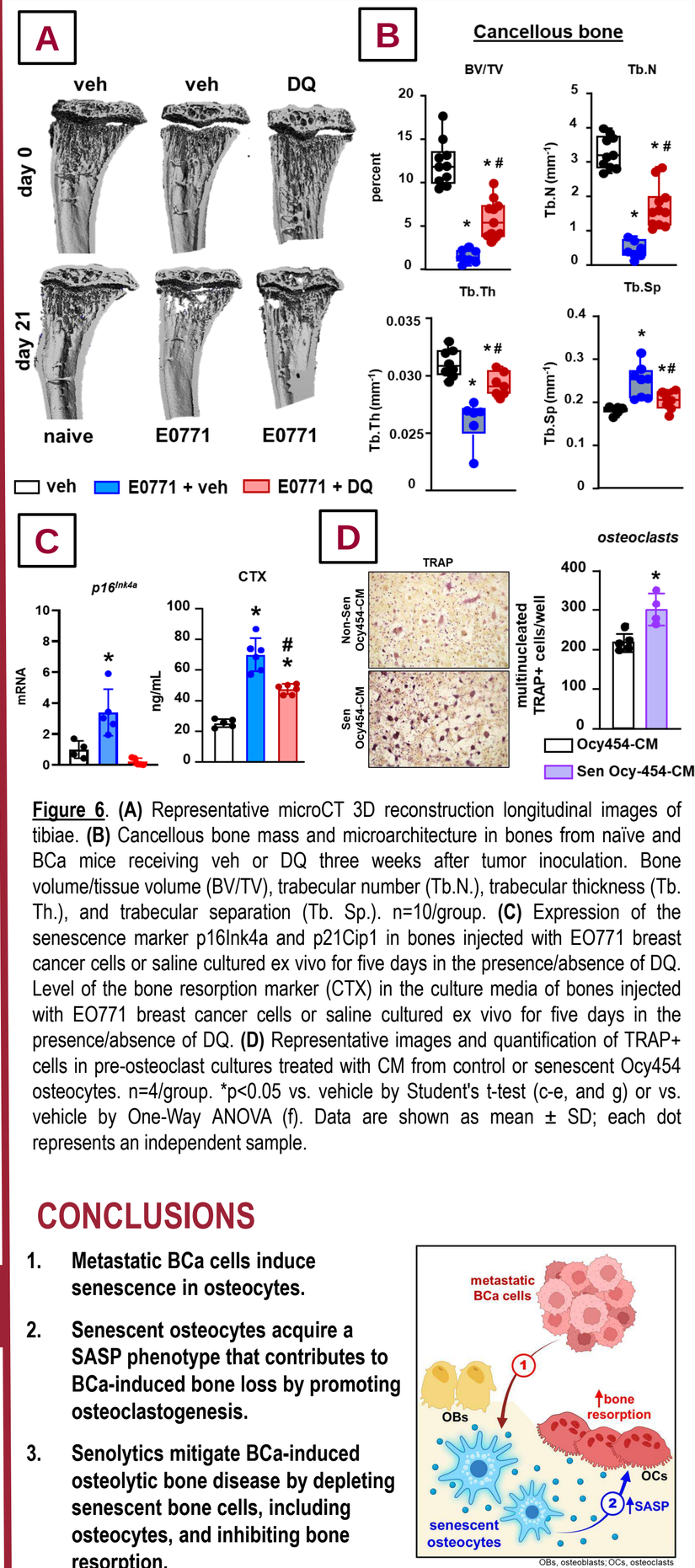

Figure 6. **(A)** Representative microCT 3D reconstruction longitudinal images of tibiae. **(B)** Cancellous bone mass and microarchitecture in bones from naïve and BCa mice receiving veh or DQ three weeks after tumor inoculation. Bone volume/tissue volume (BV/TV), trabecular number (Tb.N.), trabecular thickness (Tb. Th.), and trabecular separation (Tb. Sp.). n=10/group. **(C)** Expression of the senescence marker p16Ink4a and p21Cip1 in bones injected with EO771 breast cancer cells or saline cultured ex vivo for five days in the presence/absence of DQ. Level of the bone resorption marker (CTX) in the culture media of bones injected with EO771 breast cancer cells or saline cultured ex vivo for five days in the presence/absence of DQ. **(D)** Representative images and quantification of TRAP+ cells in pre-osteoclast cultures treated with CM from control or senescent Ocy454 osteocytes. n=4/group. *p<0.05 vs. vehicle by Student's t-test (c-e, and g) or vs. vehicle by One-Way ANOVA (f). Data are shown as mean ± SD; each dot represents an independent sample.

CONCLUSIONS

1. **Metastatic BCa cells induce senescence in osteocytes.**
2. **Senescent osteocytes acquire a SASP phenotype that contributes to BCa-induced bone loss by promoting osteoclastogenesis.**
3. **Senolytics mitigate BCa-induced osteolytic bone disease by depleting senescent bone cells, including osteocytes, and inhibiting bone resorption.**

Targeting osteocytes and their interactions with BCa cells in the TME is a new approach to improving bone health in BCa patients.

REFERENCES & ACKNOWLEDGEMENTS

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