Osteoblasts Adopt A Reversible, Non-Mineralizing Phenotype Induced By Breast Cancer Secreted Factors

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INTRODUCTION: Bone is the most common site of breast cancer metastasis, with cancer cell dissemination to bone occurring even before primary tumor diagnosis [1]. Metastatic tumors alter normal bone physiology and can cause uncontrolled bone resorption (osteolysis) leading to fracture risk along with altered hematopoiesis and immune response [2]. Biochemical factors (IL-6, PTHrP, TNFα) produced by cancer cells trigger bone resorption by osteoclasts, releasing cytokines and chemokines that drive further metastatic progression in a vicious cycle. Osteoblasts secrete factors (OPN, CXCL5) that attract cancer cells to the bone microenvironment, though the changes that cancer cells induce in osteoblasts are not well established. Cancer cells induce an inflammatory response [3] and disrupt arrangement of osteoblasts [4] therefore may modify the osteoblast phenotype (cancer-associated osteoblasts) contributing to altered bone physiology during metastasis. Whether cancer-associated osteoblasts (CAO) are recruited before or after osteogenic differentiation and if this phenotype can be rescued has yet to be investigated. This study sought to identify an altered osteoblast phenotype induced by cancer paracrine signaling in 3D coculture by 1) measuring changes in osteoblast gene expression and protein secretion and 2) quantifying osteoblast mineralization before and after a mineralizing phenotype is established.

METHODS: MC3T3-E1 murine osteoblastic cells (MC3, ATCC) were expanded and embedded at 2×10^6 cells/mL in gelatin, nano-hydroxyapatite (nHA) hydrogels cross-linked with microbial transglutaminase. 4T1 triple negative murine mammary cancer cells (4T1, ATCC) were expanded and embedded in a separate hydrogel with the same formulation. MC3T3 hydrogels were cultured in osteogenic media (50 μM ascorbic acid, 10 mM β-glycerophosphate, and 100 nM dexamethasone) alone (CTL; N=4) or in the same well as a separate 4T1 hydrogel as segregated co-cultures (N=4) for 7 days. Media was collected on day 7, and protein expression was measured using a cytokine array (Proteome Profiler Mouse XL Cytokine Array, R&D Systems). RNA was isolated on day 7 for bulk sequencing. RNA-seq results were analyzed using STAR alignment, DeSeq2, and Ingenuity Pathway Analysis. Additional MC3 hydrogels were cultured for 28 days alone (MC3), in constant coculture with 4T1 hydrogels (MC3+4T1), alone and supplemented daily with 4T1 conditioned media (MC3+4T1 CM), alone for 14 days before adding a 4T1 hydrogel constant coculture for an additional 14 days in a delayed coculture (MC3+4T1 DC), and in constant coculture for 14 days before removing the 4T1 hydrogel and culturing alone for an additional 14 days in an abbreviated coculture (MC3+4T1 AC) (Fig. 1A). Mineral deposition was quantified on days 7, 14, 21, and 28 using a colorimetric assay. Live-dead staining was performed on day 28 to validate cell viability and quantify apoptotic rates in all groups. FITC and DAPI immunofluorescent staining was performed on day 28 to analyze MC3T3 morphology.

RESULTS: Bulk RNA sequencing of osteoblasts indicated an altered transcriptome in the presence of cancer cells (Fig. 1B). There were over 6,000 differentially expressed genes between osteoblasts cultured in monoculture and in continuous coculture with cancer cells. Notably, mineralization associated genes such as collagen type 1 (COL1A1), alkaline phosphatase (ALPL), osterix (SP7), and bone morphogenic protein 2 (BMP2), were all downregulated in cancer-associated osteoblasts. Ingenuity pathway analysis (Qiagen) showed that collagen biosynthesis, integrin cell surface interactions, and bone mineralization signaling pathways were downregulated in CAO (Fig. 1C). Cancer cells also expressed an altered transcriptome in the presence of osteoblasts, with over 800 differentially expressed genes (not shown), though these changes were not required to induce changes in the osteoblasts as shown by our conditioned media cultures. The protein array detected 23 of 111 differential regulation of several osteoblast-related proteins in cancer cocultures, including increased amphiregulin, decreased IGFBP-5 and decreased periostin/osteoblast specific protein 2 (OSF-2) (Fig. 1E). Interestingly, protein levels of osteopontin (OPN) and osteoprotegrin (OPG) were unchanged. These results are corroborated by lack of mineralization in our osteoblast hydrogels when cocultured with cancer cells in both continuous coculture and conditioned media (Fig. 1F). In a delayed coculture, where osteoblast hydrogels were allowed to mineralize for 14 days alone before cancer hydrogels were added, mineralization was varied, with some samples having mineral levels similar to day 14 controls (mid-mineralization period) and others similar to day 21 controls (end of mineralization period). In abbreviated coculture, where cancer hydrogels were removed after 14 days of coculture and osteoblasts were cultured alone for a further 14 days, mineralization was rescued and calcium levels were similar to day 14 control samples.

DISCUSSION: Cancer associated osteoblasts have a non-mineralizing phenotype induced by breast cancer secreted factors. They are characterized by the downregulation of genes associated with bone mineralization and lower expression of differentiation and mineralization proteins. MC3T3 osteoblasts do not mature and deposit mineral in the presence of cancer cells, and conditioned media cultures verify that this is due to paracrine signaling from cancer cells independent of osteoblast induced changes in the cancer cell transcriptome. Actively mineralizing osteoblasts are not fully arrested by cancer coculture, with some constructs continuing to mineralize while others halted or slowed down mineralization after cancer cells were introduced. Notably, the non-mineralizing osteoblast phenotype induced by cancer cells is reversible when cancer cells are removed. Taken together, these results show that a cancer-associated osteoblast phenotype does not support mineralization, which could be an important contributor to metastatic bone loss.

SIGNIFICANCE/CLINICAL RELEVANCE: Bone metastases are associated with breast cancer mortality. We found that cancer cells alter osteoblast mineral deposition, which can contribute to metastatic progression and bone deterioration.

REFERENCES: [1] Braun et al., N Engl J Med, 2000. [2] Breuer et al., Cell Death & Dise, 2019. [3] Kinder et al., Exp Cell Research, 2008. [4] Kimura et al. Scientific Reports, 2017.

ACKNOWLEDGEMENTS: Notre Dame Naughton Accelerator Grant, NIH RO1CA252878, DOD BCRP W81XWH2110432, MIMETic IRCLA/2017/217

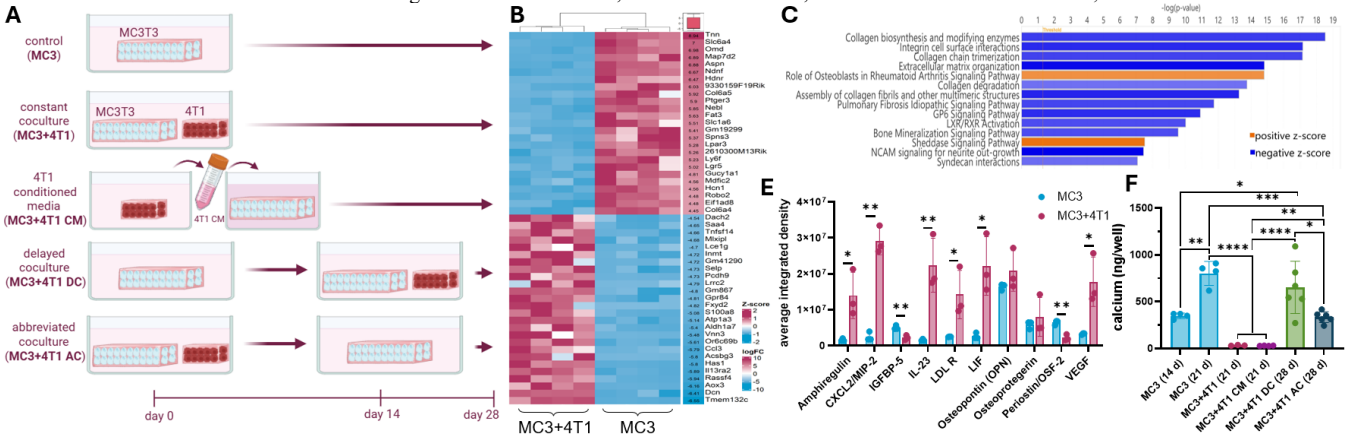

Figure 1: A) Experimental design. B) Top 25 differentially expressed genes in osteoblasts on day 7 of constant coculture of osteoblasts with cancer cells (MC3+4T1) and control (MC3). C) Top 14 significantly affected pathways with activation (orange) or deactivation (blue) z-scores. E) Protein expression levels between MC3 and MC3+4T1 groups after 7 days of culture for 10 selected proteins. F) Mineral content in osteoblast hydrogels after 14, 21, or 28 days in all groups.