

EphA2 is a clinically relevant target for breast cancer bone metastatic disease

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Abstract

EphA2 receptor tyrosine kinase (RTK) is highly expressed in breast tumor cells across multiple molecular subtypes and correlates with poor patient prognosis. As metastasis of breast cancer to bone is a major cause of morbidity and mortality in patients, we investigated the potential role of EphA2 in this clinically relevant phenomenon. Here, we demonstrate EphA2 function in breast cancer cells promotes osteoclast activation and development of osteolytic bone disease. Blocking EphA2 function molecularly and pharmacologically in breast tumors reduced the number and size of bone lesions and the degree of osteolytic disease in intratibial and intracardiac models, which correlated with a significant decrease in the number of osteoclasts at the tumor-bone interface. EphA2 loss of function in tumor cells impaired osteoclast progenitor differentiation in co-culture, which is mediated, at least in part, by reduced expression of IL-6. Expression of *epha2* mRNA is enriched in human breast cancer bone metastatic lesions relative to visceral metastatic sites, and we detected EphA2 protein expression in breast tumor cells in bone metastases in patient samples, supporting the clinical relevance of our findings. These data provide a strong rationale for development and application of molecularly targeted therapies against EphA2 for the treatment of breast cancer bone metastatic disease.

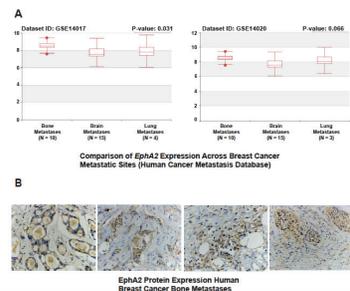


Fig. 1: A. EphA2 expression is higher in breast-to-bone metastatic lesions relative to other metastatic sites (Human Cancer Metastasis Database [<https://hcmdb.i-sanger.com/>]). **B.** EphA2 is primarily expressed in tumor cells within breast-to-bone metastatic lesions in human patient samples.

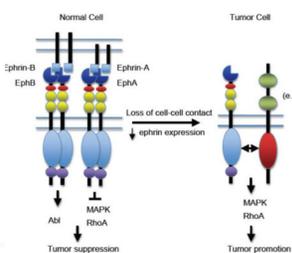


Fig. 2: Model of physiologic and pathogenic Eph family signaling. Vaught et al, *Breast Cancer Res.* 2008

RESULTS

EphA2 knockdown in tumor cells reduces osteoclast numbers in bone lesions

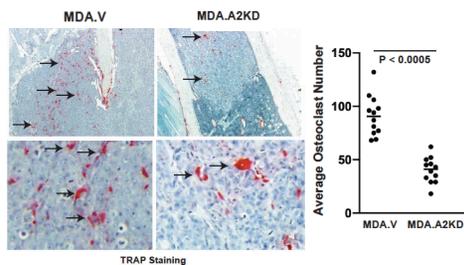


Fig. 4: A. Sections from tumor-bearing bones were stained for tartrate resistant acid phosphatase (TRAP) and the number of TRAP+ osteoclasts (arrows) were enumerated. Bone lesions produced by MDA.A2KD cells contained significantly fewer TRAP+ osteoclasts at the tumor-bone interface than bone lesions produced by control cells. N = 8 animals per condition analyzed in two independent experiments. No significant differences in the %Ki67+ tumor cell nuclei (proliferation) or Cl. Caspase 3+ tumor cell nuclei (apoptosis) were detected (data not shown).

EphA2 loss of function in tumor cells impairs osteoclast differentiation in co-culture

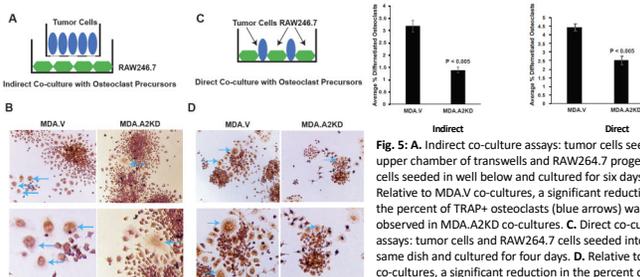


Fig. 5: A. Indirect co-culture assays: tumor cells seeded in upper chamber of transwells and RAW264.7 progenitor cells seeded in well below and cultured for six days. **B.** Relative to MDA.V co-cultures, a significant reduction in the percent of TRAP+ osteoclasts (blue arrows) was observed in MDA.A2KD co-cultures. **C.** Direct co-culture assays: tumor cells and RAW264.7 cells seeded into the same dish and cultured for four days. **D.** Relative to MDA.V co-cultures, a significant reduction in the percent of TRAP+ osteoclasts (blue arrows) was observed in MDA.A2KD co-cultures. N = 5 to 8 fields/condition from 3 independent experiments.

EphA2 in tumor cells mediates osteoclast differentiation in part by regulation of IL-6

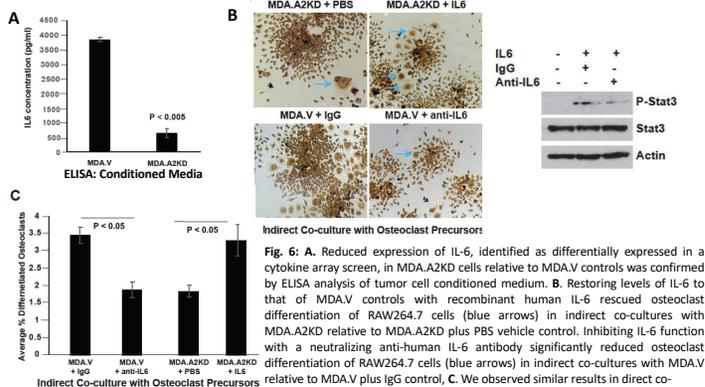


Fig. 6: A. Reduced expression of IL-6, identified as differentially expressed in a cytokine array screen, in MDA.A2KD cells relative to MDA.V controls was confirmed by ELISA analysis of tumor cell conditioned medium. **B.** Restoring levels of IL-6 to that of MDA.V controls with recombinant human IL-6 rescued osteoclast differentiation of RAW264.7 cells (blue arrows) in indirect co-cultures with MDA.A2KD relative to MDA.V plus PBS vehicle control. Inhibiting IL-6 function with a neutralizing anti-human IL-6 antibody significantly reduced osteoclast differentiation of RAW264.7 cells (blue arrows) in indirect co-cultures with MDA.V relative to MDA.V plus IgG control. **C.** We observed similar results in direct co-culture assays (data not shown). N = 5 to 8 fields/condition from 3 independent experiments. **(D)** Activity of recombinant human IL-6 and neutralizing activity of anti-human IL-6 antibody was confirmed in immunoblots for Stat3 activation in RAW264.7 cells. Recombinant IL-6 induced Stat3 phosphorylation, and phosphorylation was inhibited in RAW264.7 cells stimulated with IL-6 plus neutralizing anti-IL6 antibody. Uniform loading was confirmed by probing blots for total Stat3 and actin.

RESULTS

Pharmacologic inhibition of EphA2 reduces osteolytic disease *in vivo*

A Intracardiac Injection Model

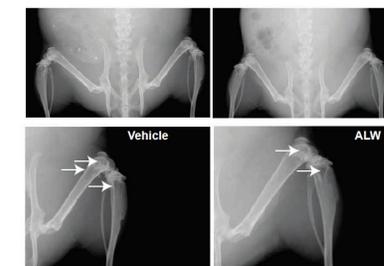
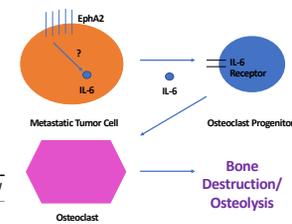
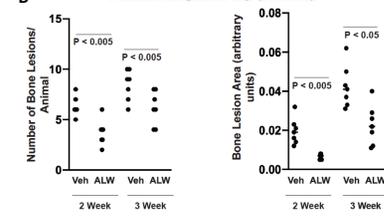


Fig. 6: We injected MDA-MB-231 human metastatic breast cancer cells into the hearts of nude female mice. One week after injection, we treated animals twice daily with EphA2 tyrosine kinase inhibitor ALW-41-27 (ALW) or control vehicle. **A.** After three weeks, we observed osteolytic bone lesions in several bones (arrows), which we measured using morphometric software (Image J) and enumerated. **B.** Relative to vehicle control, ALW-treated animals displayed a significant reduction in the number and size of osteolytic lesions relative to vehicle control at both two weeks and three weeks post tumor injection. **C.** Model of one mechanism by which EphA2 appears to facilitate breast tumor growth and osteolysis in the bone microenvironment.

B Faxitron Digital X-ray (3 Week)



CONCLUSIONS

EphA2 expression is enriched in breast-to-bone metastatic lesions relative to other metastatic sites

EphA2 is expressed primarily on tumor cells in the bone microenvironment

Genetic or pharmacologic inhibition of EphA2 reduces osteolysis in breast cancer bone metastatic lesions, in part through reduced expression of IL-6, in both MDA-MD-231 xenografts and 4T1 allografts (4T1 data not shown)

EphA2 is a clinically relevant target for breast cancer patients with bone metastatic disease

FUTURE DIRECTIONS

Determine the role of ephrin ligands and direct cell contact on tumor cell-osteoclast interactions – known that reverse signaling induced by ephrin-A2 on osteoclast progenitors suppresses osteoblast differentiation on osteoblast progenitors expressing EphA2 in normal bone homeostasis. Similar role in cancer?

Determine the role of other EphA2-regulated cytokines that stimulate osteoclast differentiation and the molecular mechanisms through which EphA2 regulates expression of these cytokines

Determine the impact of tumor EphA2 functions on other components of the bone microenvironment (osteoblasts, osteocytes, hematopoietic progenitors, endothelial cells, immune cells)

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