Reciprocal Activation of Breast Cancer Micrometastases and the Lung Epithelium During Metastatic Outgrowth

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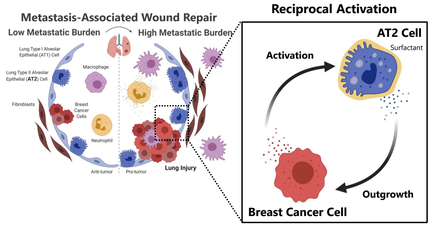
Introduction

- The lung is one of the most common sites of breast cancer (BC) metastasis.
- Triple-negative breast cancers (TNBC) preferentially metastasize to the lung.
- The overall prognosis for BC patients diagnosed with metastatic disease is 2-3 years. Patients diagnosed specifically with lung metastases have a poorer prognosis of approximately 1 year.
- No metastasis-specific therapeutic strategies are available to effectively treat patients with metastatic disease.
- Tumors are known to alter the surrounding microenvironment.
- Preparation of the metastatic niche/microenvironment, prior to metastasis, is known to promote metastatic colonization.
- ***How established micrometastases remodel the lung microenvironment and how this contributes to metastatic outgrowth is not well understood.***
- We have identified an association between aberrant lung wound healing and metastatic outgrowth that has identified tangible targets for intervention.

Hypothesis

We hypothesize that BC lung micrometastases activate surrounding lung epithelial cells which, in turn, support the survival/outgrowth of metastases within the lung.

Overall Goal



The overall goal of these studies is to identify factors secreted by lung resident cells that could be used as lung metastasis-specific therapeutic targets/agents.

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Methods

Mouse Models of BC Metastasis

- MMTV-PyMT (mouse mammary tumor virus-polyoma middle T antigen) transgenic mice: Develop spontaneous mammary tumors that metastasize to the lung.
- Late-stage metastasis model: Met-1 mouse mammary carcinoma cells, originally isolated from a MMTV-PyMT primary tumor, were injected into the tail veins of immunocompetent, syngeneic FVB mice.

Metastasis-Associated Wound Repair Analysis

Custom multispectral fluorescent imaging panels were used to quantify metastasis-associated wound repair and type II alveolar epithelial (AT2) cell activation in the lung directly adjacent to metastases

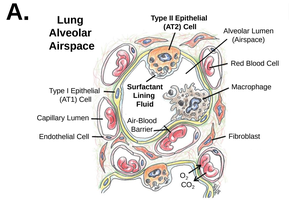
Single Cell RNA-sequencing (scRNAseq)

Lungs from mice with a low or high metastatic burden, using the late-stage metastasis model, were dissociated and RNA expression was measured for individual cells by scRNAseq. Approximately 3,000 cells per sample were sequenced with a read-depth of ~125,000 reads/cell via the 10X Genomics platform and Illumina NovSeq 6000 platforms at the University of Colorado's Genomics and Sequencing Core. Read mapping and expression quantification were performed using a combination of the 10X Cellranger pipeline and custom analytic scripts. AT2 cell changes in gene expression: upregulation of pro-proliferative/anti-apoptotic factors *Spp1* (secreted phosphoprotein 1; encodes osteopontin, Opn), *Lcn2* (lipocalin 2), *Lgals1* (galectin 1) and downregulation of the anti-tumor AT2-specific factor *Sftpb* (surfactant protein B; encodes SP-B); see Fig. 3B.

Co-Culture Model

Human A549 lung carcinoma cells were cultured for >8 passages to shift them into a more epithelial AT2 cell phenotype. Human iPSC (induced pluripotent stem cells) were differentiated to lung AT2 cells. Lysotracker was used to stain AT2-specific lamellar bodies to verify differentiation. No contact co-culture experiments were performed for 5-8 days, and TNBC cell numbers were measured using the Crystal Violet cell viability assay.

Indicators of wound repair are more abundant in the lung surrounding large versus small metastases



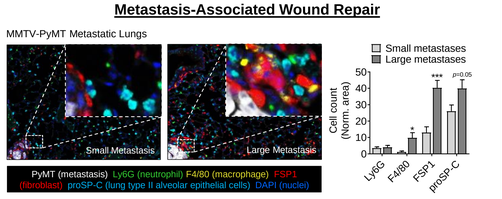
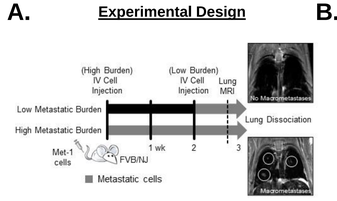
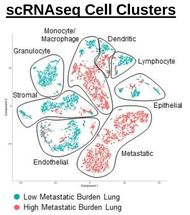


Figure 1. (A) Illustration of the cellular components and key features of lung alveoli; adapted from Harkema *et al.* 2019. **(B)** MMTV-PyMT lung metastases were separated by size (Small: diameter <150μm and Large >300μm) and stained by multispectral immunofluorescent (IF) imaging; 20X objective, inset zoom 5X. Adjacent cell counts (within 100μm distance) were normalized to area (n=8 fields from 2-4 mice/group). Mean ± SEM; * $p<0.05$, *** $p<0.001$.

An activated, inflammatory microenvironment is present in lungs with a high metastatic burden





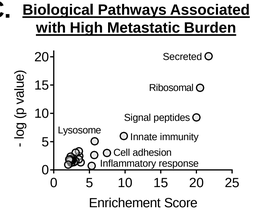
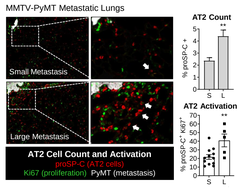


Figure 2. (A) Model of late-stage lung metastasis single cell RNA-sequencing (scRNAseq) experiment (n=1 mouse/group) using MMTV-PyMT-derived Met-1 mouse mammary carcinoma cells injected intravenously (IV). MRI of lungs prior to sequencing confirmed metastatic burden. Lung macrometastases have been circled. **(B)** Single cell gene expression data with cells clustered by gene expression. Cell types were defined by the most highly expressed genes per cluster. **(C)** Overall gene expression pathway analysis of the top significantly altered genes per sample using DAVID tool.

Metastasis-adjacent lung type II alveolar epithelial (AT2) cells are activated during metastatic outgrowth



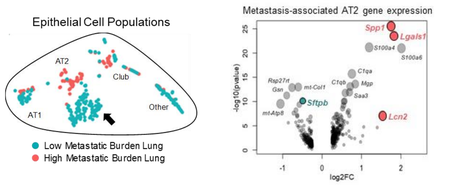


Figure 3. (A) MMTV-PyMT metastatic lungs co-stained for proSP-C, Ki67 and PyMT using multispectral IF imaging. The percentage of proSP-C+ AT2 cells (% of total cells) and proSP-C+Ki67+ activated AT2 cells (arrows, % of total AT2 cells) were quantified. 20X objective, inset zoom 5X; mean ± SEM, ** $p<0.001$, n=5 mice. S, small metastases; L, large metastases. **(B)** scRNAseq (Figure 2) epithelial cell clusters defined by the top 3-5 genes expressed per cluster (arrow showing AT2 cells) and differences in gene expression in AT2 cells from lungs with a low versus high metastatic burden.

Ecrg4 is associated with aggressive micrometastases

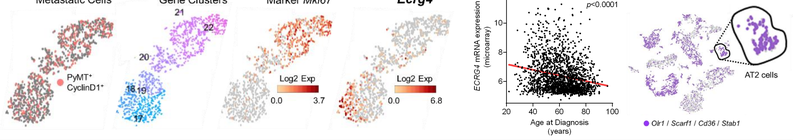
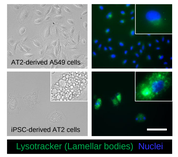
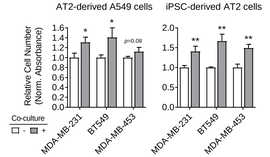
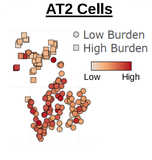


Figure 4. (A) scRNAseq of mice with metastatic lungs. The metastatic cell cluster was defined by co-expression of *PyMT* and *CyclinD1*. Metastatic cells were further segregated by gene expression into six clusters. Expression of *Mki67* was used to identify proliferative metastatic cells. Expression of the metastasis-specific gene *Ecrg4* (esophageal cancer-related gene 4; encodes that secretory molecule augurin). **(B)** Young age at diagnosis is associated with high *ECRG4* levels in BC patients. Data was obtained from the METABRIC publically available dataset. **(C)** Lung AT2 cells express at least one of four established Ecrg4 receptors by scRNAseq.

Changes in the AT2 secretome promote TNBC growth







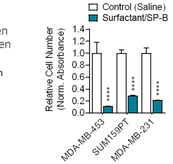


Figure 5. (A) Human AT2 cell models for studying the effects of AT2-derived factors on human TNBC cells. Lysotracker was used to verify AT2 cell status by staining AT2-specific lysosome-like lamellar bodies. Scale bar = 50μm, inset zoom 4x; iPSC, induced pluripotent stem cells. **(B)** TNBC cell growth increased following no-contact co-culture with human AT2 cells for 5-8 days; mean ± SD, * p<0.05, ** p<0.01. **(C)** *Sftpb* gene expression was significantly downregulated in AT2 cells during metastatic outgrowth in mouse lungs. **(D)** Surfactant/SP-B (surfactant protein B) supplementation with Infasurf for 5 days inhibited TNBC cell growth; mean ± SD, **** p<0.0001.

Conclusions and Future Directions

- Aberrant wound repair develops during metastatic outgrowth.
- Lung AT2 cells adjacent to growing metastases become activated, which is characterized by significant pro-tumor alterations to their secretome.
- BC metastasis-secreted Ecrg4 may influence AT2 signaling in the metastatic lung.
- AT2 secreted factors may reciprocally promote metastatic tumor cell growth.
- **Summary: Our studies demonstrate that targeting the lung microenvironment, in addition to directly targeting malignant cells, may be an effective way to treat and manage BC lung metastases.**
- Future studies will continue to elucidate how activated resident lung cells support metastatic progression and could lead to the development or repurposing of therapeutic strategies to prevent destructive metastatic outgrowth.
  - Infasurf is an FDA-approved drug made from naturally-derived calf lungs for the treatment of premature infants with pulmonary surfactant deficiencies.

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