Reciprocal Activation of Breast Cancer Metastases 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) frequently metastasize to the lung.
- The overall prognosis for BC patients diagnosed with metastatic disease is 2-3 years; those with lung metastases have an average prognosis of only 1 year.
- BC cells metastasize to the peripheral tissue of the lung, commonly to the lung parenchyma where alveoli are a central component.
- Type II alveolar epithelial (AT2) cells are the most numerous cells within the alveolus and are likely to interact extensively with metastatic cells in the lung.
- **How established micrometastases remodel the lung microenvironment and how this contributes to metastatic outgrowth is not well understood.**
- We identified a connection between metastatic outgrowth and aberrant lung wound healing that has revealed multiple tangible targets for intervention.

Hypothesis

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

Overall Goal

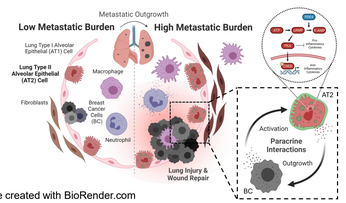


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The overall goal of these studies is to identify factors secreted by lung epithelial cells that could be targeted to reduce metastatic outgrowth and thereby improve patient survival and quality of life.

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 female FVB/NJ mice.

Single Cell RNA-sequencing (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 (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.

Co-Culture Model

Human A549 lung carcinoma cells were cultured for >8 passages to shift them into a more epithelial AT2-like phenotype. Human iPSC (induced pluripotent stem cells) were differentiated into lung AT2 cells (iAT2). Lysotracker was used to stain AT2-specific lamellar bodies to verify differentiation. No contact co-culture experiments were performed for 5-7 days.

Indicators of wound repair and AT2 cell activation develop in the lung during metastatic outgrowth

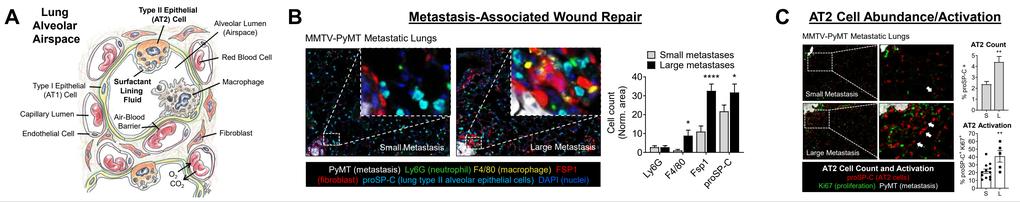


Figure 1. (A) Illustration of the key features of the lung alveoli; adapted from Harkema *et al.* 2019. **(B)** MMTV-PyMT lung metastases stained by multispectral immunofluorescent (IF) imaging. Adjacent cell counts (within 300μm) were normalized to area, n=16 metastases from 2-4 mice per group. **(C)** MMTV-PyMT metastatic lungs co-stained for proSP-C, Ki67 and PyMT. The percentage of proSP-C+ AT2 cells (as a % of total cells) and proSP-C+Ki67+ activated AT2 cells (white arrows, as a % of total AT2 cells) were quantified; n=17 metastases from 3-5 mice per group. Mean ± SEM; * p<0.05, ** p<0.01, **** p<0.0001.

Paracrine interactions between lung AT2 cells and BC cells

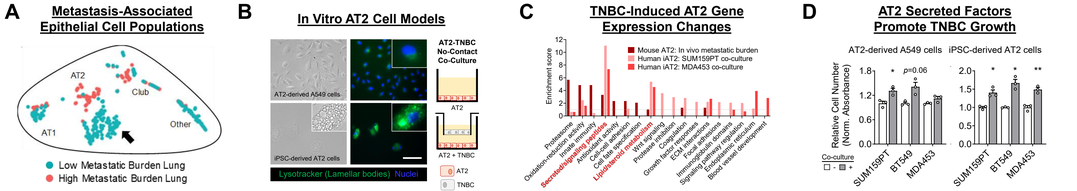


Figure 2. (A) scRNAseq comparing lungs with a low (1 week post-injection) vs high (3 weeks post-injection) metastatic burden (n=1 mouse/group) using MMTV-PyMT-derived Met-1 mouse mammary carcinoma cells injected intravenously (IV). **(B)** Human AT2 cell models for studying the reciprocal effects of secreted factors on human AT2 and TNBC cells. Scale bar = 50μm; iPSC, induced pluripotent stem cells. **(C)** Bulk RNAseq was performed on AT2 cells no-contact co-cultured for 3 days with SUM159PT and MDA-MB-453 (MDA453) TNBC cells. Pathways analysis comparison of all three AT2 RNAseq datasets. **(D)** TNBC cell growth following no-contact co-cultured with human AT2 cells for 5-7 days, normalized to cells cultured alone; mean ± SEM, * p<0.05, ** p<0.01.

BC cells induce changes to AT2-derived pulmonary surfactant composition, while exogenous surfactant supplementation inhibits BC cell viability

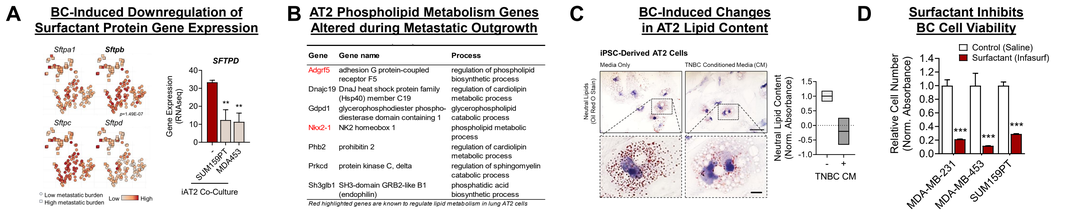


Figure 3. (A) Surfactant protein (*Sftp*) gene expression differences in AT2 cells from the lung scRNAseq dataset and surfactant protein D (*SFTPD*) gene expression in AT2 cells no-contact co-cultured with TNBC cells from the bulk RNAseq dataset. **(B)** Phospholipid metabolism genes are altered in mouse AT2 cells during metastatic outgrowth. **(C)** AT2 cells were cultured in media alone or a 50% dilution of conditioned media (CM) from SUM159PT cells for 3 days. Neutral lipid content was measured by Oil Red O stain; scale bar = 50μm, inset scale bar = 10μm. Lipid stain was quantified as relative absorbance; median. **(D)** TNBC cell viability in a 50% dilution of the FDA-approved pulmonary surfactant Infasurf for 5 days; normalized to control cells. Mean ± SD, *** p<0.001.

BC-induced lung inflammation and CREB Signaling

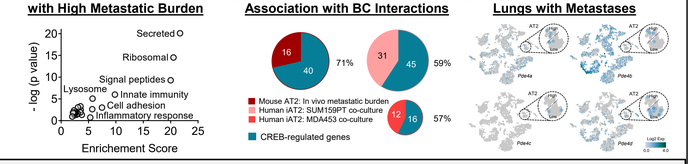


Figure 4. (A) scRNAseq gene expression data from all lung cell types combined by condition (low versus high metastatic burden) indicating the biological pathways most highly associated with metastatic outgrowth. **(B)** The proportion of AT2 secreted factor genes enriched following BC interactions that are CREB (cAMP-responsive element-binding protein)-regulated for all three AT2 RNAseq datasets, percentage indicated. **(C)** t-SNE visualizations of cells from metastatic mouse lungs clustered by gene expression and colored by *Pde4* (phosphodiesterase 4) isoform expression.

Targeting BC growth with the anti-inflammatory PDE4 inhibitor Roflumilast

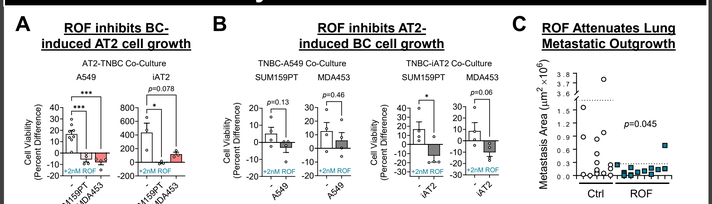


Figure 5. (A) AT2 cell viability was measured by crystal violet assay following co-culture with TNBC cells and treatment with 2nM roflumilast (ROF) for 5-7 days. **(B)** TNBC cell viability was measured by crystal violet assay following co-culture with AT2 cells and treatment with 2nM ROF for 5-7 days. **(C)** Following metastatic colonization with Iv injected Met-1 mouse mammary carcinoma cells (n=5-8 mice per group), mice were administered the FDA-approved anti-inflammatory drug roflumilast (ROF). The number and size of metastases were measured per mouse. The dotted lines represent the mean per group based on serial section quantifications. Mean ± SEM; * p≤0.05, *** p<0.001.

Conclusions and Future Directions

- Metastatic outgrowth is associated with a wound repair-like response in the adjacent lung.
- AT2 cells adjacent to growing metastases become activated and undergo significant alterations to their secretome.
- AT2 secreted factors promote tumor cell growth.
- AT2 pulmonary surfactant protein and lipid levels are altered by BC-derived secreted factors and surfactant supplementation inhibits TNBC cell viability.
- Metastasis-associated wound repair and AT2-induced tumor cell proliferation can be reduced by the anti-inflammatory PDE4 inhibitor roflumilast.
- **Summary: Our studies demonstrate that targeting lung epithelial cells in the metastatic microenvironment may reduce progression of BC lung metastases.**
- Future preclinical studies will test whether repurposing FDA-approved lung-specific interventions, in combination with standard of care agents, are viable strategies to prevent destructive metastatic outgrowth within the lung in preparation for future clinical trials.