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14. ABSTRACT Metastatic dissemination is the leading cause of cancer death. Each year in the United States more than 500,000 people die principally as a result of the metastatic spread of cancer. This project aims to revisit the century-old "seed and soil" hypothesis and suggests a new paradigm for the role of stromal cells in metastasis. It is commonly accepted that only cancer cells travel from primary to metastatic sites. I hypothesize that, together with cancer cells, stromal cells of the primary tumor travel to the secondary site and provide provisional stroma until the metastatic cells recruit new stroma in the secondary organ. In this report we show that host-derived cells can escape primary tumors along with cancer cells, survive in blood circulation, and multiply in metastatic nodules. Pre-existence of a tissue-like structure in the form of heterotypic cell fragments may increase the viability of cancer cells in blood circulation and at the secondary site. Immunohistochemical analysis suggests that host-derived cells found at the metastatic site are myofibroblasts.					
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Introduction

This project aims to revisit the century-old “seed and soil” hypothesis and suggests a new paradigm for the role of stromal cells in metastasis. It is commonly accepted that only cancer cells travel from primary to metastatic sites. But some of the genes that distinguish highly metastatic from non-metastatic tumors are specifically expressed by stromal cells (eg, fibroblasts, immune cells). This suggests that host stromal cells may alter the metastatic potential of tumors. Furthermore, aggregates with tumor cells, stromal cells and extracellular matrix have been found in blood and lymph vessels around primary tumors. I hypothesize that, together with cancer cells, stromal cells of the primary tumor travel to the secondary site and provide provisional stroma until the metastatic cells recruit new stroma in the secondary organ.

Body

Year 1 of this training award focused on identifying the composition of cell aggregates that are shed by tumors, determining the viability of shed cells and cell aggregates and characterization of metastasized host cells. The major goals as outlined in the newly submitted SOW were: i) setting up a isolated tumor perfusion model in mice expressing ubiquitously the green fluorescent protein (GFP, *Actb-GFP/C57BL* mice); and ii) develop skin transplants from *Actb-GFP/C57BL* mice to wild type mice (*C57BL/6*mice) using parabiosis, and implant metastatic tumors in the transplanted skin. Both are designed to study the role of primary tumor stromal cells in spontaneous metastasis to lung.

Identifying the composition of cell aggregates that are shed by tumors and determining the viability of shed cells and cell aggregates.

I implanted DsRed-expressing metastatic lung cancer cells (LLC1) in the kidneys of *Actb-GFP/C57BL* mice, and used the isolated tumor perfusion model to collect the efferent blood (Fig. 1A)(1). Single cancer cells, homotypic-cell fragments, and fragments of tumors containing both stromal and cancer cells were detected in the perfusate from the tumor 10–12 days after inoculation (Fig. 1B). Approximately 81% of the shed cancer cells and fragments were single cells, while the rest were tumor fragments (up to 150 μ m in size). All the large fragments (composed of 6 or more cells) contained GFP-expressing host cells (Figs. 1B, C). These tumors shed an average of 129 ± 71 (mean \pm SD) heterotypic-cell fragments/hr/g tissue.

To evaluate the viability of shed cancer cells, I used the Carboxyfluorescein Caspase 3 FLICA. Non-transgenic *C57BL/6* mice were inoculated with *DsRed*-LLC1 cells at the kidney and the shed cells were collected. I detected caspase 3 and 7 activity (detected as green fluorescence signal) in dsRed⁺ cancer cells by confocal microscopy and evaluated the ratio of apoptotic cells in the single cancer cells and 2-3 cell clumps and compared it with cancer cell apoptosis in cell clumps containing more than 6 cells (Fig. 2). At the time of shedding, caspase 3 and 7 activation—a measure of cell apoptosis—was detectable in most of the single or doublets of cancer cells, but significantly less in the cancer cells within heterotypic-cell fragments (Fig. 2).

Characterization of metastasized host cells.

To characterize the nature of the primary site-derived host cells, I used the parabiotic mouse model (Fig. 3) to perform skin transplantation. *Actb-GFP/C57BL* mice—that had previously received restorative bone marrow transplantation from non-transgenic mice—were conjoined to establish parabiosis (2) with non-transgenic mice. After one month, mice were separated and a skin flap (2–4 cm in diameter) from the GFP-transgenic mouse remained in the wild-type mouse. This procedure allowed transplantation of skin containing GFP⁺ cells in mice with no detectable blood-circulating GFP⁺ cells, measured by flow cytometry. LLC1 cells were implanted subcutaneously in the transplanted area, allowed to grow up to a diameter of 10 mm, and then primary tumors were excised to allow metastases to grow. GFP⁺ host-derived cells were detectable in lung metastases in all five animals in which the primary tumor contained GFP⁺ host cells (Fig 4). Unfortunately dsRed expression in most of the LLC1 used in this experiment was lost; nevertheless I was able to identify metastatic regions in the lung based on morphology (high cellularity) using DAPI staining. To characterize the origin of the GFP⁺ cells found in the micrometastases in the lung, tissue sections were stained with α SMA (a marker for

myofibroblasts), F4/80-Cy3 (a murine macrophage marker), or Sca-1-Cy5 (stem cell antigen-1) (Fig 5A-C). Using the confocal microscope I found co-localization of the “green” stromal cells and α SMA positive cells suggesting that some of these stromal cells were myofibroblasts. The additional staining with F4/80 and Sca-1 did not co-localize with the “green” stromal cells, indicating these are not macrophages nor stem cells.

Breast Cancer Training Program.

With respect to the longitudinal clinic and coursework described in the proposed breast cancer training program, I completed a clinical course in the MGH Department of Radiation Oncology on radiation biology as it pertains to clinical treatment and a course at the Health Science and Technology division of MIT and Harvard Medical School, entitled “Endothelial cells in health and disease”. I also attended the course “Critical Issues in Tumor Microcirculation, Angiogenesis and Metastasis” at Harvard Medical School. In Year 2, I will begin shadowing physicians in the breast clinic.

Key Research Accomplishments:

Developed an isolated tumor perfusion model

Developed a Actb-GFP skin transplant model using parabiosis

Reportable Outcomes:

No reportable outcomes at this time as defined by the reporting procedures.

A paper is in preparation regarding the Actb-GFP skin transplant as a method for studying stromal cells in spontaneous tumor metastases.

Conclusions:

Host-derived cells can escape primary tumors along with cancer cells, survive in blood circulation, and multiply in metastatic nodules. Pre-existence of a tissue-like structure in the form of heterotypic cell fragments may increase the viability of cancer cells in blood circulation and at the secondary site. Immunohistochemical analysis suggests that host-derived cells found at the metastatic site are myofibroblasts.

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2. A. J. Wagers, R. I. Sherwood, J. L. Christensen, I. L. Weissman, *Science* **297**, 2256 (Sep 27, 2002).

Figures:

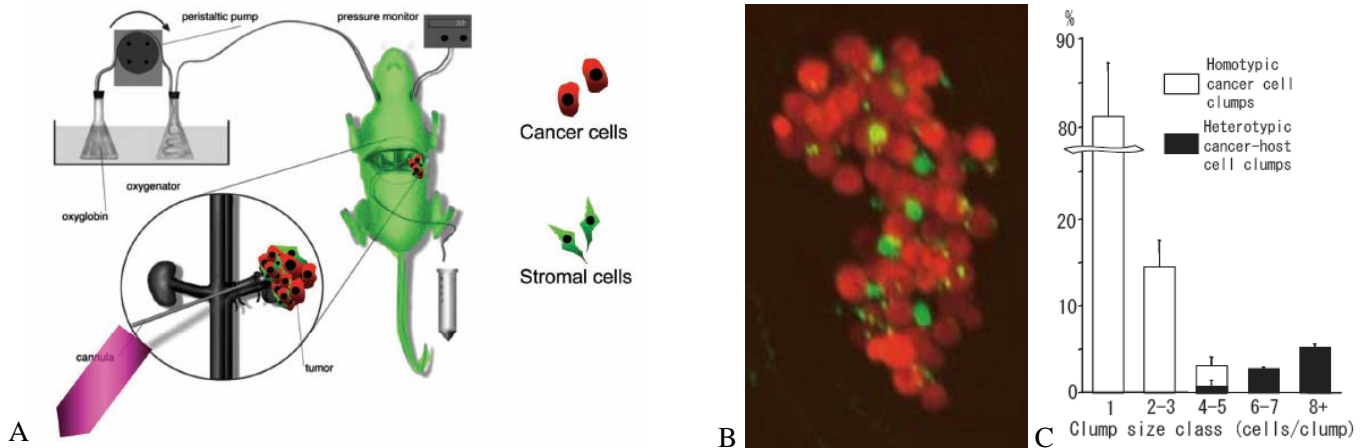
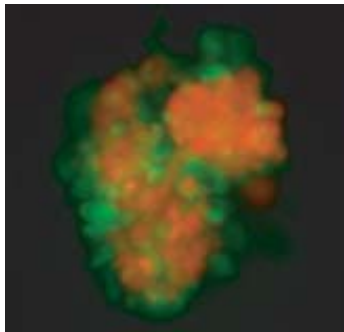
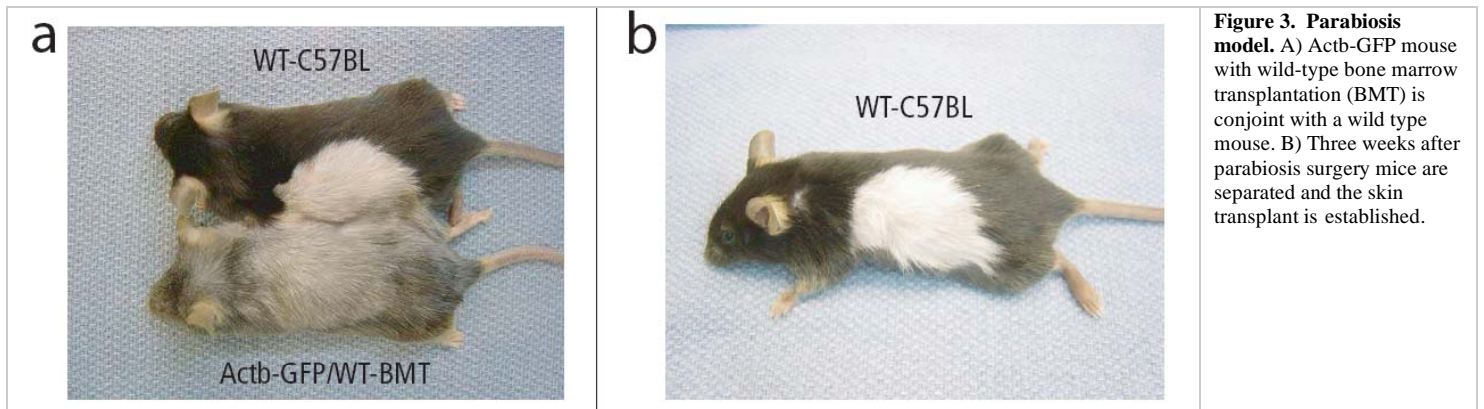


Figure 1. Kidney tumor perfusion model (A) and shed tumor and stromal cells (B, C). (A) Shed tumor cells (DsRed+, Red) and stromal cells (GFP +, green) were collected from the renal vein of *Actb-GFP/C57BL* mice bearing *DsRed-LLC* tumors in the kidney. (B) Representative multiphoton microscopy image of a shed heterotypic-cell fragment containing *DsRed-LLC1* cancer cells and GFP+ host-derived cells. (C) Histogram of shed tumor cells/fragments from *DsRed-LLC1* tumors grown in *Actb-GFP/C57BL* mouse kidneys collected over 1 hour of systemic perfusion. Host-derived GFP+ cells were present in all fragments containing 6 cells or more.



Percentage of viable cancer cells in the shed tumor cell clumps	
Single & 2-3 cell class (Homotypic)	6-7 & 8+ cell class (Heterotypic)
12.0 ± 4.1 %	22.8 ± 4.5 %*

Figure 2. Viability of the shed tumor cells. Detection of apoptotic cells in the shed fragments by caspase staining. Over 22% of the DsRed cancer cells (Red) within fragments were negative in caspase 3 and 7 activity assay (showed in green), while significantly fewer of the single or doublets of cancer cells were viable (~12%, * P < 0.05). Data are expressed as mean ± SD of four independent experiments.



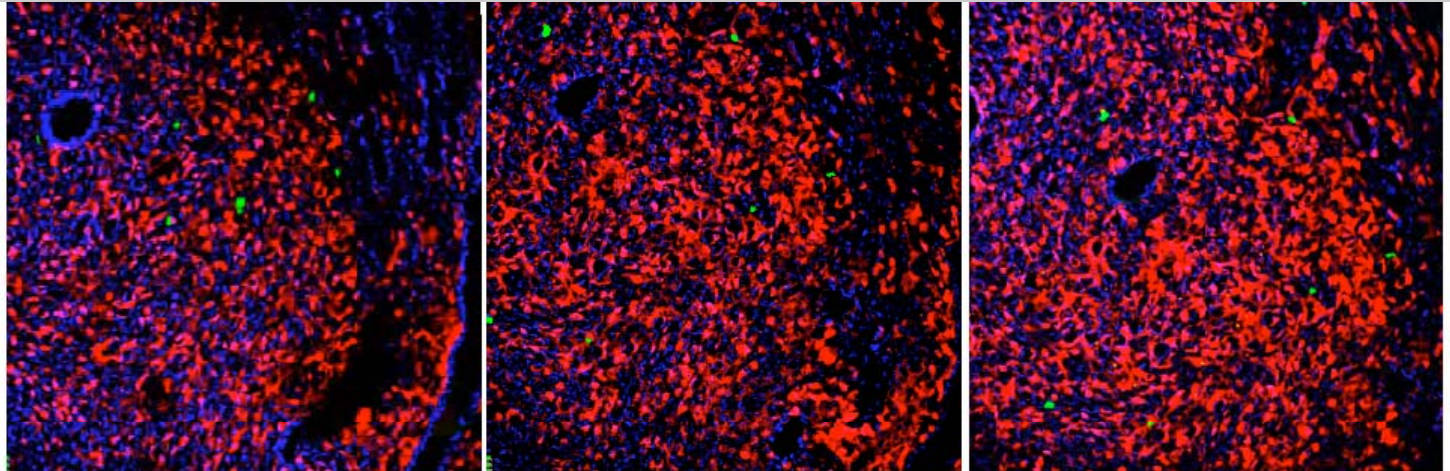


Fig 4. Traveling stromal cells in lung metastasis from dsRed tumors grown in the GFP+ skin transplant. GFP+ host cells were detected in the lung metastases from subcutaneously grown LLC1dsRed tumors (implanted in the transplanted skin area). Confocal microscopy for tumor cells (red), GFP+ host cells (green) and DAPI (blue). Images are 630 μ m across.

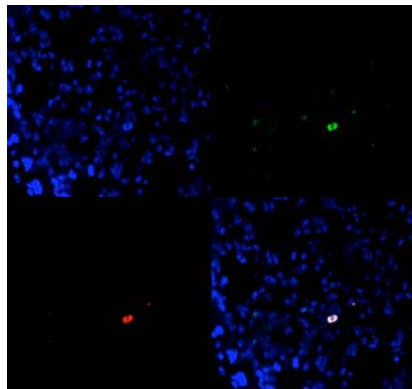


Fig 5A. Red α SMA-Cy3, Green GFP-labeled stromal cell, blue DAPI nuclear stain. α SMA-Cy3 and GFP co-localization suggests myofibroblast origin of stromal cells.

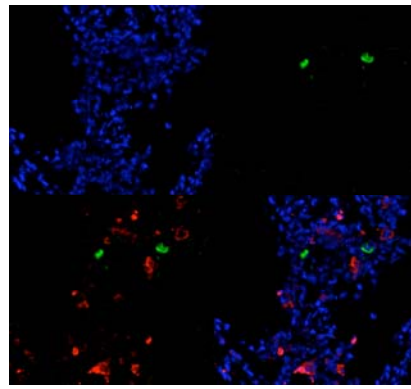


Fig 5B. Red F4/80-Cy3, Green GFP-labeled stromal cell, blue DAPI nuclear stain. F4/80-Cy3 and GFP do not co-localize excluding macrophages as a possible origin of these stromal cells.

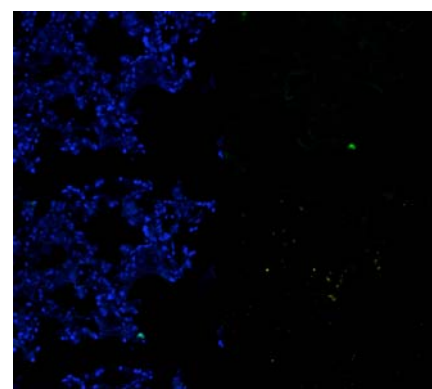


Fig 5C. Red Sca1-Cy5, Green GFP-labeled stromal cell, blue DAPI nuclear stain. Sca1-Cy3 and GFP do not co-localize, excluding mesenchymal stem cells as the origin of these stromal cells.