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14. ABSTRACT
The project focuses on the role of the local microenvironment on the escape of disseminated breast carcinoma cells from dormancy at the metastatic site and specifically, on the role that the cell surface heparan sulfate proteoglycan syndecan-1 (Sdc1) plays in these events. In a genetic model, we discovered that host Sdc1 is required for the efficient outgrowth of disseminated carcinoma cells into lung metastases. When analyzing the p38 pathway in mouse tissues, we found that this MAPK is activated in host lung tissue of Sdc1 KO mice compared to WT mice (Aim 1). The development of an in vitro model to reconstitute the metastatic niche in the lung is ongoing. We are optimizing conditions to generate vascular structures and to grow lung endothelial cells, carcinoma cells and lung fibroblasts in 3D in microfluidic devices (Aim 2). We have generated $Sdc1^{lox/lox}$ mice and confirmed the correct location of loxP sites by adenoviral transduction of fibroblasts with Cre recombinase. Expression of Cre has been confirmed in Col1a2-Cre-ER(T) Cre driver mice by Western blot in TAM-treated fibroblasts and $Sdc1^{lox}$/ Col1a2-Cre-ER(T) heterozygous hybrids have been generated (Aim 3).

15. SUBJECT TERMS
Breast neoplasms, breast cancer, metastasis, dormancy, stroma, matrix, proteoglycans, migration, invasion, lung, syndecans

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1. INTRODUCTION

Overwhelming evidence supports the notion that the dissemination of breast carcinoma cells can be an early event. One of the main reasons for the delay between dissemination and the development of overt metastatic disease is because disseminated carcinoma cells become dormant and remain dormant for extended periods of time in distant organs. Experimental observations point to the local microenvironment as a critical determinant of dormancy. Since metastatic disease overwhelmingly is the cause of death of breast cancer patients, it becomes imperative to understand the mechanisms that govern maintenance and, conversely, escape from dormancy. Our own observations suggest that syndecan-1 (Sdc1) plays an important role in the outgrowth of disseminated carcinoma cells into metastatic lesions. This proposal aims to examine the role of Sdc1 in the microenvironment using in vitro and in vivo approaches.

2. KEYWORDS

Breast neoplasms, breast cancer, metastasis, dormancy, stroma, matrix, proteoglycans, migration, invasion, lung, syndecans

3. ACCOMPLISHMENTS

Major Task 1: Analyze stromal Sdc1, ECM alignment and dormancy in lung after tail vein injection of carcinoma cells

Subtask 1: Submit documents for ACURO approvals

Completed 100%; ACURO approval has been obtained.

Subtask 2: Optimize dormancy markers

In progress (30%); phospho-p38 and total p38 have been optimized (**Fig. 1**). Phospho-Erk ½ and Ki67 optimization are in progress.

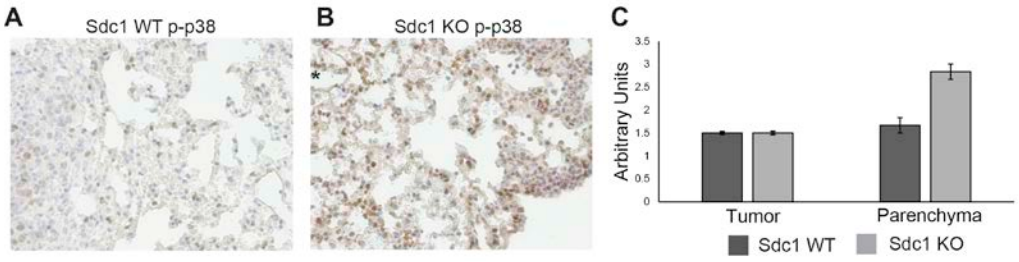

Figure 1: Loss of host Sdc1 induces activation of the p38α signaling pathway in lung tissue. Sections of tumor-bearing lung from tail-vein injection experiments were stained with an antibody to phosphorylated p38α. Levels of p-p38 were higher in host lung parenchyma of Sdc1 KO (**B**) than of Sdc1 WT (**A**) animals (p=0.043). Conversely, p-p38 levels in the 4T1 tumor cells did not differ significantly between Sdc1 WT and Sdc1 KO mice (p=0.5). (Original magnification: 100x).

We have discovered that p38 is activated in host lung tissue of Sdc1-/- mice compared to Sdc1+/+ mice. Conversely, no difference in p38 activation is detected in metastatic tumor cells growing in animals of the different genotypes. This result was unexpected. It is currently unknown whether p38 activation is causally related to decreased metastatic efficiency in Sdc1-/- animals.

Subtask 3: Perform tail vein injection experiment

In progress (30%); Lung metastases have been examined at the 15 and 30-day time points. At 30 days, the metastasis are becoming confluent and individual metastases cannot be discerned. At 15 days, distinct metastases are present but individual disseminated tumor cells were not identified. The number of metastatic lesions is significantly reduced in Sdc1-/- compared to Sdc1+/+ mice (**Fig. 2A**) and the metastases in Sdc1-/- are significantly smaller (**Fig 2B**). In future experiments, we will examine earlier time points (24h, 4d, 8d) and possibly use less aggressive murine tumor cells should we fail to detect individual disseminated tumor cells.

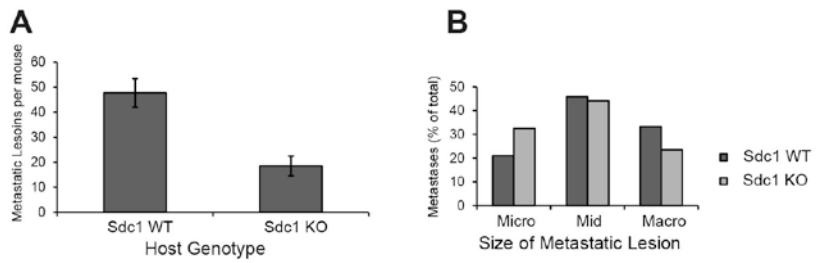

Figure 2: Loss of host Sdc1 reduces metastatic efficiency. A) 4T1 murine mammary carcinoma cells were injected via tail vein into Sdc1 WT and Sdc1 KO mice. After 15 days, animals were sacrificed and the tumor burden was analyzed. **A**) We observed a significant decrease in the number of lung metastatic lesions per mouse in Sdc1 KO mice compared to Sdc1 WT mice (p=0.0038). Furthermore, the 4T1 lesions observed in Sdc1 KO mice were significantly smaller compared to lesions observed in Sdc1 WT mice (p=0.024).

Subtask 4: Image lung sections with Nuance imaging system

Not yet started (0%); Image analysis is dependent on the identification of single disseminated tumor cells (ST 1.3).

Subtask 5: Image lung sections with multi-photon microscope for SHC signal

In progress (20%); Lung sections with metastatic lesions have been imaged by second harmonic generation microscopy. We are able to image collagen fibers in the lung sections (**Fig. 3**) but so far, the analysis has been limited to established metastases rather than individual tumor cells in the perivascular region.

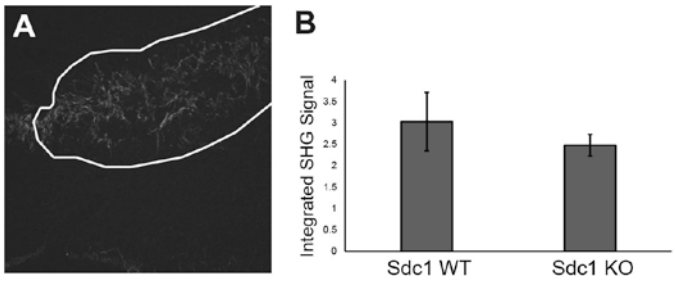

Figure 3: Imaging of fibrillar collagen in lung metastases. Unstained sections of mouse lung from tail vein injection experiments were imaged with a two-photon microscope using second harmonic generation (SHG) modality. **A**) Representative image of SHG signal from 4T1 cell lung metastatic lesion from Sdc1 WT mouse. **B**) SHG signals from metastatic lesions were integrated and are expressed as signal (arbitrary units) per area. There was no significant difference between metastases in Sdc1 WT vs Sdc1 KO mice (p = 0.36).

Subtask 6: Analysis of imaging data with inform program

Not yet started (0%); Image analysis is dependent on the identification of single disseminated tumor cells (ST 1.3).

Major Task 2: Optimize 3D lung microenvironment assay

Subtask 1: Transduce HMVEC-L with E4ORF1 and mCherry

Not yet started (0%); We have obtained HMVEC-L and murine lung endothelial cells and have made progress growing the cells in lumen structures in micro-channel devices (see below) but have not yet transduced them with E4ORF1 nor labeled them. If we succeed in generating defined vascular structures in microchannel devices, there will be no need to label the endothelial cells.

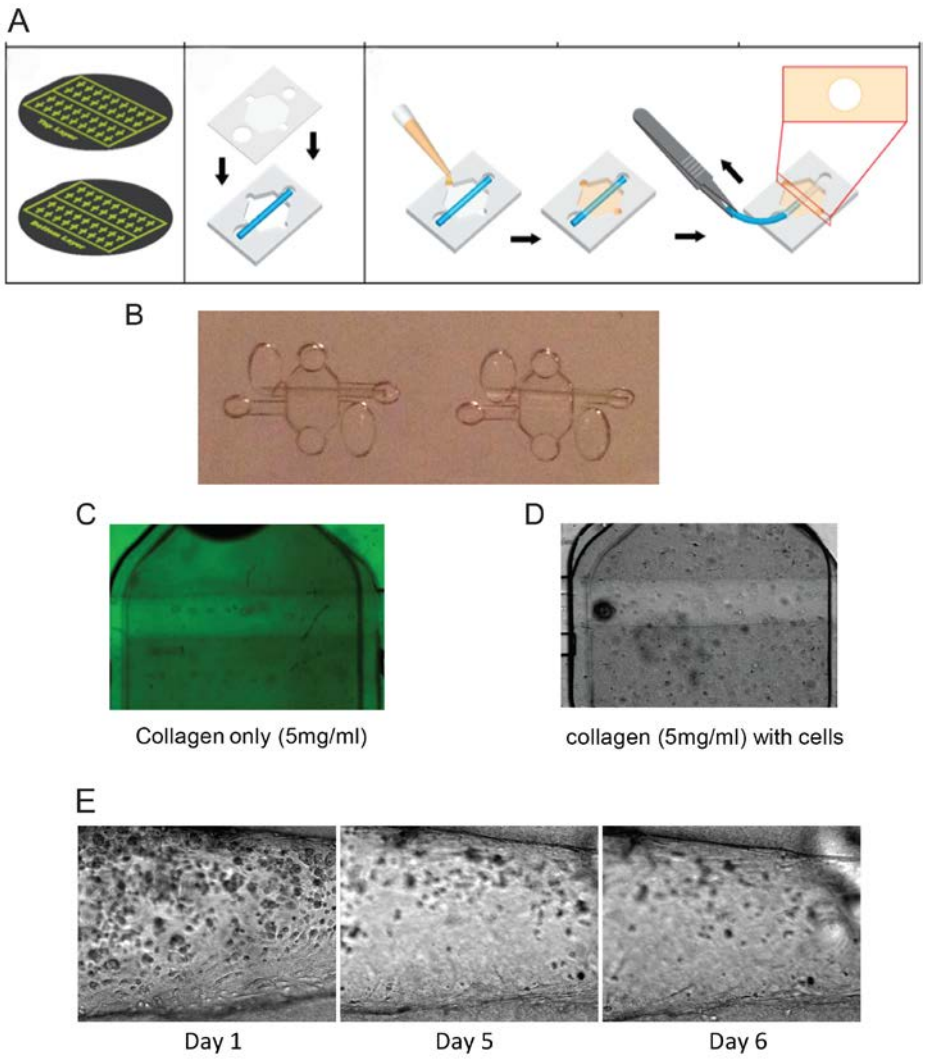

Figure 4: Modeling of the perivascular lung tumor microenvironment in microfluidic channels. A) Schematic representation of arrayed devices (left panel), individual device with space holder rod (middle panel), and procedure of filling device with collagen and creating vessel-like lumen by removing the rod (right panel). **B**) Two actual devices in arrayed configuration. **C**) Image of collagen gel after removal of rod. **D**) Collagen gel with embedded carcinoma cells. **E**) High magnification phase contrast images of lumen coated with HMVEC-L after different culture times. Lumens were coated with fibronectin and vitronectin prior to perfusion with endothelial cell suspension; growth medium contained VEGF.

Subtask 2: Fluorescently label human breast carcinoma cells

In progress (60%); We have labeled MDA-MB-231 human breast carcinoma cells and 4T1 and 4T07 murine mammary carcinoma cells with CellBright cell membrane staining dyes. We also generated GFP labeled MDA-MB-231 and T407 cells, although, the former became heavily mycoplasma contaminated and the latter lost GFP expression after approximately a month of culture.

Subtask 3: Optimize ECM production in microchannels

In progress (50%); we have focused our efforts on creating 3D vessel-like structures in collagen gels, which requires embedding of fibroblasts into collagen gels.

Subtask 4: Optimize 3D co-culture in microchannels

In progress (50%); We are in the process of optimizing growth of lung endothelial cells (HMVEC-L, LMVEC), carcinoma cells and fibroblasts in 3D collagen in microfluidic devices. In collaboration with Dr. David Beebe, Department of Biomedical Engineering, we are examining the feasibility of reconstituting the perivascular lung microenvironment in arrayed microchannel devices. To create a vessel-like structure, a flexible rod is embedded in a collagen I gel (**Fig. 4A**). After the gel has solidified, the rod is removed and the lumen is perfused with an endothelial cell suspension. Lung fibroblasts and carcinoma cells can be embedded into the collagen matrix.

We found that primary human lung endothelial cells (HMVEC-L) line the lumen structures and remain viable for extended periods of time (tested up to 7 days) when the collagen-lined lumens are coated with fibronectin and vitronectin (**Fig. 4E**). Lung fibroblasts and carcinoma cells remain viable in collagen gels (5 mg/ml). In addition to embedding carcinoma cells into the collagen gels, we will be able to seed carcinoma cells into the vessel-like structures and observe their fate in the peri-vascular environment after trans-endothelial migration.

Subtask 5: Optimize dormancy marker labeling and imaging in microchannels

Not yet started (0%)

Major Task 3: Perform 3D microenvironment co-culture experiments

Subtask 1: Perform 3D microenvironment experiments with Sdc1 mutants

Not yet started (0%)

Subtask 2: Perform 3D microenvironment experiments with inhibitors

Not yet started (0%)

Major Task 4: Generate tumor-bearing, fibroblast-specific, conditional Sdc1 knock-out mice

Subtask 1: Submit documents for ACURO approvals

Completed 100%; ACURO approval has been obtained.

Subtask 2: Cross Sdc1loxP/loxP mice with Cre-ER(T) mice

In progress (25%); After crossing Sdc1 loxP/loxP mice with homozygous Cre-ER(T) mice, we have obtained heterozygous hybrids. We are currently having difficulties with the reliable analysis of the

Cre genotype. Once these PCR issues are solved, we will continue breeding and generate Sdc1 loxP/loxP-Cre-ER(T) +/+ mice.

Subtask 3: Cross MMTV-PyMT mice with Cre-ER(T) mice Sdc1loxP/loxP mice

Not yet started (0%)

Major Task 5: Perform metastasis experiments with PyMT-Sdc1cKO mice

Not yet started (0%)

What opportunities for training and professional development has the project provided?

The in vivo experiments associated with this project including tail vein injection metastasis assays and the generation of conditional Sdc1 KO mice was conducted by Colleen Chute, a graduate student in the Friedl lab and a member of the Tumor Biology program of the University of Wisconsin (McArdle Institute). Ms. Chute successfully defended her thesis entitled “Host Syndecan-1 And Metastatic Efficiency Of Mammary Carcinomas” on August 18, 2015. She continues on in the Friedl lab as postdoctoral fellow.

How were the results disseminated to communities of interest?

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?

The focus during the next reporting period will be on the following activities:
1. Develop a method to identify single disseminated carcinoma cells in lung tissue.
2. Perform tail vein injection experiments with earlier time points to detect individual disseminated carcinoma cells in lung tissue.
3. Optimize additional dormancy markers in mouse tissue (p-Erk1/2, Ki67)
4. Identify p-p38-positive cells in host lung tissue with co-localization experiments.
5. Complete optimization of lung microenvironment assay in arrayed microfluidic devices.
6. Optimize dormancy marker analysis in microfluidic devices.
7. Continue breeding of conditional Sdc1 KO mice to generate homozygous Sdc1 loxP/loxP-Cre-ER(T) +/+ animals.
8. Cross MMTV-PyMT mice with Cre-ER(T) mice Sdc1loxP/loxP mice.

4. IMPACT

Nothing to report

5. CHANGES/PROBLEMS

Changes in approach and reasons for change

A minor change in approach affects Major Task 2 (Optimize 3D lung microenvironment assay). Instead of modeling the perivascular niche by co-seeding fibroblasts, carcinoma cells and endothelial cells in a “flat” culture system, we have worked on creating vessel-like structures in 3D in microfluidic devices. We believe that the vessel-like structures represent a higher fidelity model of the perivascular niche. The arrangement of the devices in an array will allow us to test a variety of conditions.

Actual or anticipated problems or delays and actions or plans to resolve them

The optimization of some of the dormancy markers in mouse tissues has been delayed due to turn-around time issues in the TRIP (Translational Research in Pathology) core laboratory. We anticipate that the work associated with this project will receive a higher priority in the near future. Alternatively, we will perform the stain optimizations in the Friedl lab.

The optimization of the PCR reactions to determine the Cre genotype (Cre-ER(T)) has been challenging and has delayed breeding of the Sdc1 loxP/loxP-Cre-ER(T) +/+ hybrid animals. We anticipate that these technical issues will be resolved in the near future.

Changes that had a significant impact on expenditures

Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report at the present time. We are considering isolating primary lung endothelial cells and fibroblasts from cadaver tissue. We will seek approval from the IRB and submit an ACURO amendment application if we plan to go ahead with this plan.

6. PRODUCTS

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name: Andreas Friedl, M.D.
Project role: PI
No change

Name: Ning Yang, Ph.D.
Project role: Associate Scientist
No change

Name: Colleen Chute, Ph.D.
Project Role: Postdoctoral Fellow (since August 2015, before Research Assistant, aka Graduate Student)
Nearest person month worked: 12
Contribution to Project: Dr. Chute is responsible for all in vivo experiments associated with this project and she performs most of the tissue-based assays.
Funding Support: no other support

Name: Emmanuel Sampene, Ph.D. (replacement for Sandeep Saha, M.S. who has left the institution)
Project Role: Assistant Researcher, 0.6 cal mos,
Nearest person month worked: 1
Contribution to Project: Dr. Sampene is responsible for all statistical analyses throughout the course of the project
Funding Support: UWCCC cancer center core grant and other individual investigator grants.

Name: Kristy Meyer, B.S.
Project role: Research Specialist
No change

Kevin Eliceiri, B.S.
Project role: Collaborative support for advanced imaging, including collagen imaging by SHG.
No change

8. **SPECIAL REPORTING REQUIREMENTS**

Nothing to report

9. **APPENDICES**

None