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TITLE: Regulation of Metastatic Breast Cancer Dormancy

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14. ABSTRACT Metastasis is a multistep process whereby cells from the primary tumor undergo an epithelial to mesenchymal transition (EMT) allowing for dissemination into metastatic niches such as the brain, bone and liver. Once attaining the metastatic organ the rate-limiting step in metastasis is that the cells must survive in a new niche and proliferate to form a frank metastasis. For reasons that are still incompletely understood, many breast cancer cells can remain dormant for years to even over a decade before proliferating into a distant macrometastasis. To begin to understand this important knowledge gap we have developed an all-human hepatic bioreactor. In this award period we have established that the hepatic bioreactor is functional for 30 days by functional and injury markers (BUN, AST, ALT, CYP). We have generated micrometastases in the bioreactor and determined that breast cancer cell lines enter spontaneous dormancy in the bioreactor. We have also completed pilot experiments in mouse models for spontaneous metastasis formation with breast cancer cell lines.					
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1. INTRODUCTION:

Metastasis is a multistep process whereby cells from the primary tumor undergo an epithelial to mesenchymal transition (EMT) allowing for individual cell motility to disseminate and eventually extravasate into common metastatic niches such as the brain, bone and liver. Once attaining the metastatic organ a significant rate-limiting step occurs; these metastatic cells must survive in a new niche and proliferate to form a frank metastasis. For reasons that are still incompletely understood, many breast cancer cells can remain dormant for years to even over a decade before proliferating into a distant macrometastasis. *We propose that phenotypic reversion to a more epithelial phenotype allows for this seeding and subsequent dormancy.*

This novel postulate is based on findings that small metastases express E-cadherin and other epithelial markers absent from the primary tumor mass. Previous studies in our laboratory have shown that this shift is likely caused by metastatic microenvironment as co-culturing metastatic breast carcinoma cells with hepatocytes causes re-expression of E-cadherin in metastatic breast cancer cells. Interestingly, E-cadherin re-expression during co-culture with hepatocytes also confers chemotherapeutic resistance to metastatic breast cancer cells. As yet, the role of the epithelial reversion in carcinoma seeding at metastatic sites and dormancy is undefined. *We hypothesize that the epithelial phenotype is required for breast cancer seeding at metastatic sites and entrance into dormancy.*

2. KEYWORDS: Metastasis, Epithelial-to-Mesenchymal Transition, Dormancy

3. ACCOMPLISHMENTS:

■ What were the major goals of the project?

SA1. Determine whether the epithelial phenotype allows for cell seeding, survival and dormancy in the liver.

Task 1. Determine required partial epithelial reversion for cell seeding and dormancy in a soft-walled all human bioreactor. (months 1-24)

1a. Engineer tagged MCF7 cells with shRNA against E-cadherin and engineer tagged MDA-MB-361 and MDA-MB-231 cells to express or prevent expression of E-cadherin (months 1-6) **100% completed**

1b. Seed rat hepatocytes into soft-walled human bioreactor with MCF7, MDA-MB-361 and MDA-MB-231 cells and optimize E-cadherin detection, cell seeding numbers and time points. (months 1-9) **100% completed**

1c. Seed human hepatocytes into soft-walled human bioreactor with parental and engineered MCF7, MDA-MB-361 and MDA-MB-231 cells and track E-cadherin expression. (months 6-18) **90% completed**

Task 2. Determine required partial epithelial reversion necessary for cell seeding and dormancy in an *in vivo* model of breast cancer. (months 1-24)

2a. Pilot experiment to optimize time points for sacrifice of mice (20 mice total) for metastatic dissemination with MDA-MB-231 cells. (Months 6-18) **75% completed**

2b. Inoculate mice in the mammary fat pad with MCF7_v, MCF7shE, MDA-MB-231_v, MDA-MB-231shE or MDA-MB-231Ecad, MDA-MB-361_v, MDA-MB-361shE or MDA-MB-361Ecad (shE: shRNA against E-cadherin; Ecad: E-cadherin inducible expression; v: vector alone). Each treatment group will contain 15 mice, 5 will be sacrificed at each time point (pre-metastatic, micrometastatic and macrometastatic; total mice 120).

Primary tumors will be measured with calipers and target organs, primary tumor and blood will be assayed for tumor cells and E-cadherin (and other markers) expression levels. (Months 12-30) **0%**

SA2. Determine the pattern of epithelial marker expression in metastatic cell seeding and dormancy in the liver using primary human tumor specimens.

Task 1. Assay E-cadherin expression in primary breast cancer cells undergoing cell seeding and dormancy in a soft-walled all human bioreactor. (months 12-36) **10% completed**

1a. Optimize de-identified primary breast cancer cell seeding with rat hepatocytes in the bioreactor. (months 12-24) **10% completed**

1b. Seed primary breast cancer cells and human hepatocytes into the bioreactor and track E-cadherin (and other markers) expression through breast cancer cell seeding and dormancy. (months 18-36) **0%**

Task 2. Determine required partial epithelial reversion necessary for deidentified primary breast cancer cell seeding and dormancy *in vivo*. (months 12-36)

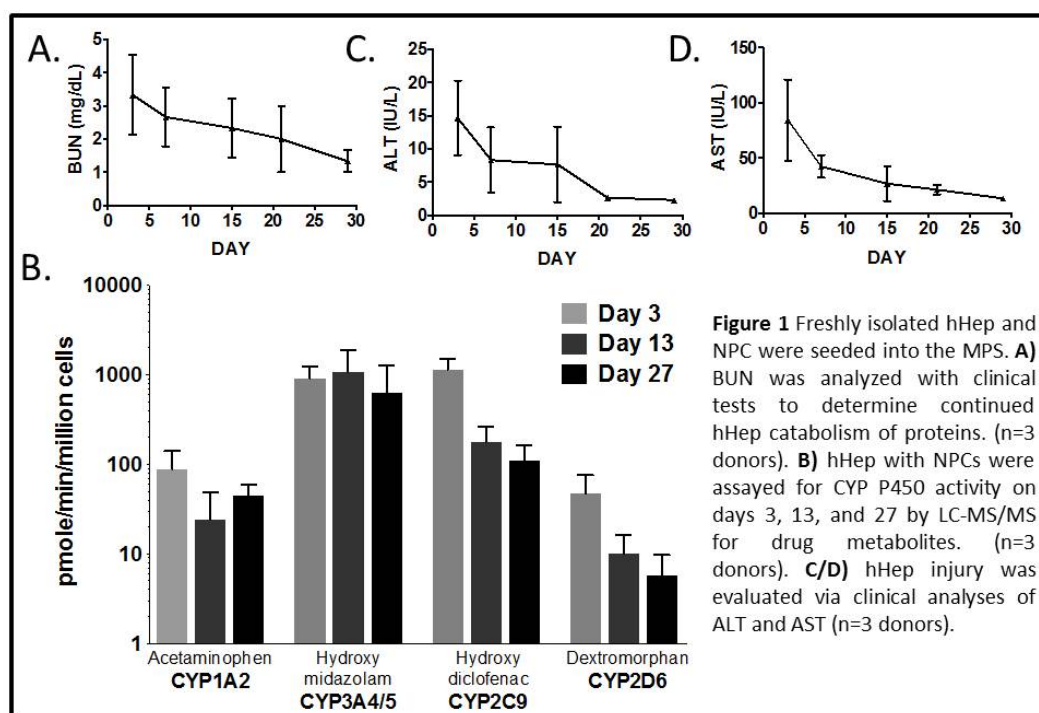
- 2a.** Optimize *in vivo* growth of de-identified primary patient samples. Inoculate mice with varying cell numbers in the mammary fat pad and sacrifice one mouse each week to check for metastasis progression. We anticipate this will only require 10 mice but this is dependent on the heterogeneity of the patient tumors. Primary tumor and target organs will be evaluated for metastasis, E-cadherin and other markers. (months 12-30) **10% completed**
- 2b.** Inoculate mice with de-identified primary patient samples (n=10). Each treatment group will contain 9 mice, 3 will be sacrificed at each time point (pre-metastatic, micrometastatic and macrometastatic; total mice 90). Measure tumors with calipers target organs, primary tumor and blood will be assayed or tumor cells and E-cadherin (among other makers) expression levels. (months 21-36) **0%**

▪ **What was accomplished under these goals?**

I. Specific Aim 1 Tasks 1a-c

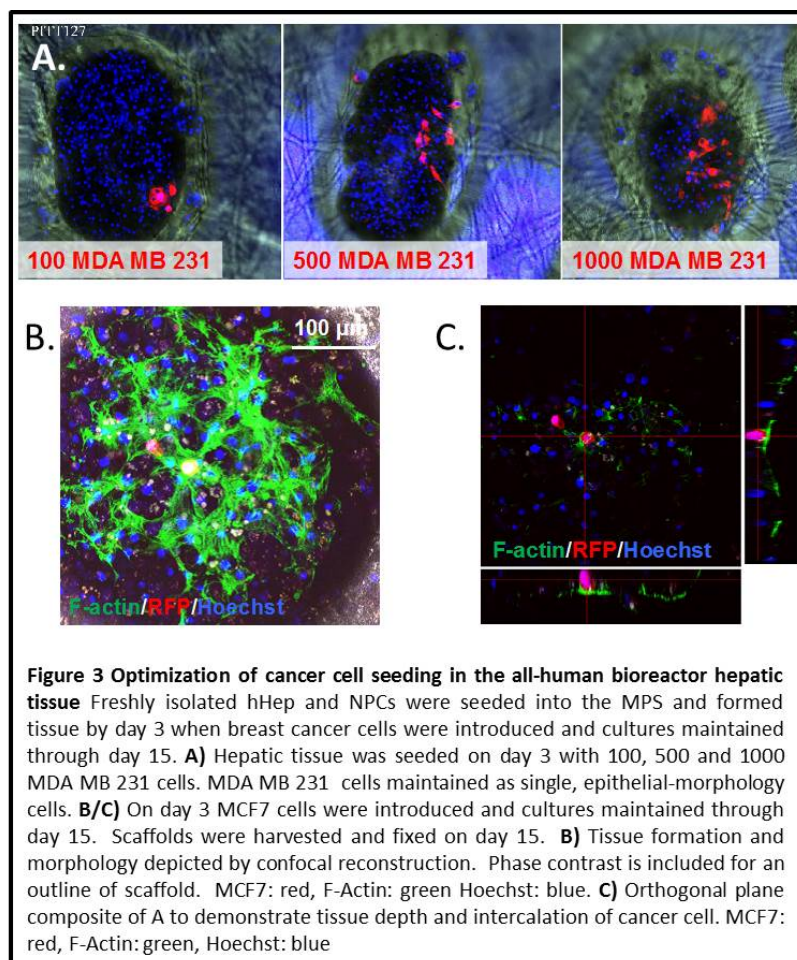
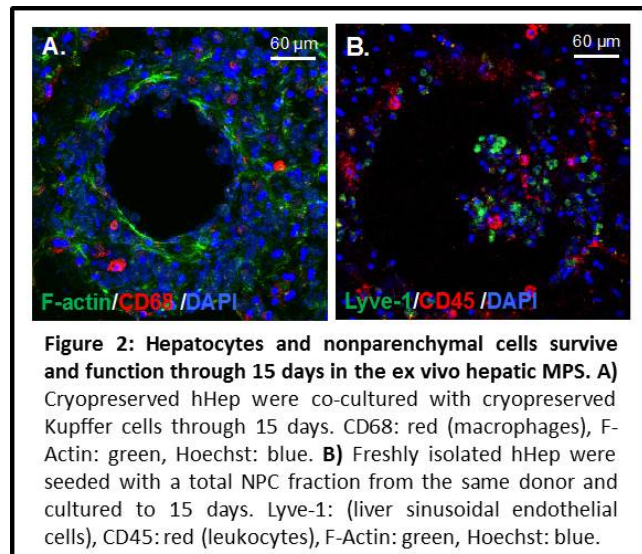
Model system establishment and validation

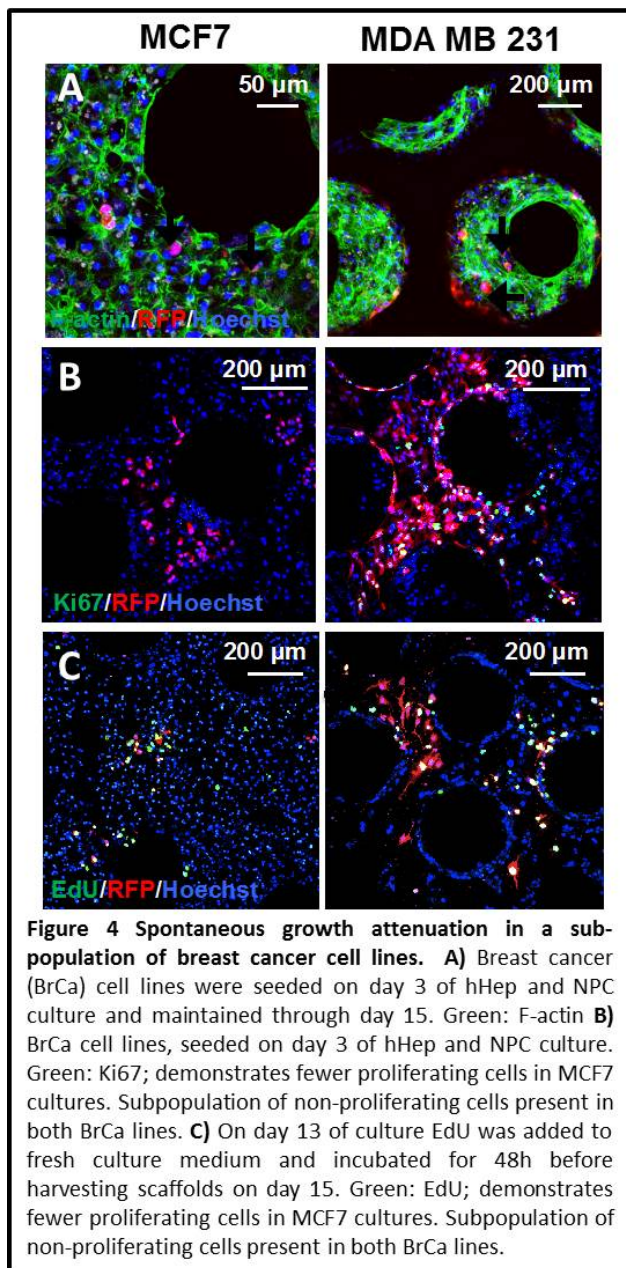
SA1 Task 1a (100% completed): I have acquired the cell lines MDA MB 231, MCF7, and MDA MB 361 and engineered with RFP. MDA MB 231s have been engineered with shRNA against Ecadherin as well as an Ecadherin construct. MCF7s have been engineered with shRNA against Ecadherin (these are natively high Ecadherin expressors). MDA MB 361's are still being engineered due to the slow growth rate this line is taking months to select for stable Ecadherin shRNA and expression.



SA1 Task 1b (100% completed) and 1c (80% completed): I validated the soft-walled all human hepatic bioreactor through 30 days of culture. This was important to verify that I could establish functional hepatocytes and non-parenchymal cells (NPCs) through necessary timepoints. I determined that the appropriate quality controls needed to include both function and injury markers. Hepatocyte functional assays include blood urea nitrogen (BUN) run in a CLIA approved clinical laboratory which detects continued catabolism of proteins by hepatocytes (Figure 1A). Cytochrome P450 (CYP) function is also vital for hepatocytes to metabolize endogenous and exogenous compounds (one of the primary functions of these cells). I assayed for the function of 4 different CYPs in collaboration with a neighboring laboratory. I run a cocktail of compounds over the hepatocytes for 1 hour and a neighboring laboratory (Dr. Venkataraman) analyzes the metabolites of these compounds by LC MS/MS (Pillai et al J Pharm Biomed Anal. 2013 Feb 23; 74:126-32) (Figure 1B). I assessed hepatocyte injury with the AST and ALT clinical assays. These are commonly used assays for liver injury, both

are intracellular enzymes released into circulation on hepatocyte injury (Figure 1C and D). I have also evaluated the nonparenchymal cells via imaging on day 15. I confirmed the presence of macrophages, leukocytes and liver sinusoidal endothelial cells (Figure 2A and B). Overall the system was robust through 30 days of culture with continued hepatocyte function. Injury was decreased after the initial tissue formation. Nonparenchymal cells were present through day 15 and further analysis at day 30 is underway.





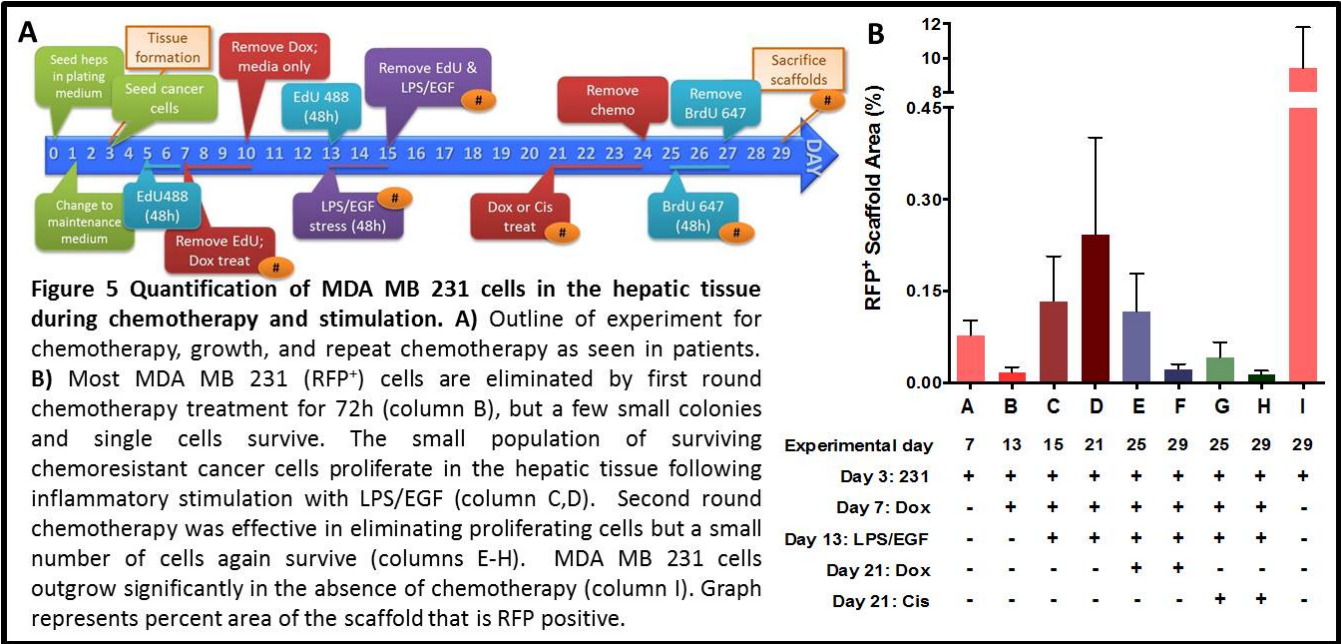
conditions for further seeding into the bioreactor and inoculation into mice

Breast cancer cell lines and dormancy in the all-human hepatic bioreactor

SA1 Task 1b (100% completed) and 1c (90% completed): I optimized seeding of breast cancer cell lines tagged with RFP into the all-human hepatic bioreactor on day 3 of culture (Figure 3A). Imaging of the breast cancer cells revealed that despite being in the bioreactor system for 12 days many of these cells remained as single cells (Figure 3B and C). I assessed the growth status of the single cells in MCF7 and MDA MB 231 in the all-human hepatic bioreactor to demonstrate dormancy (Figure 4A). A significant subpopulation of the cells underwent spontaneous growth attenuation as noted by Ki67 staining (Figure 4B) and a 48 hour incubation with EdU (Figure 4C). I also found that MCF7 had a higher number of non-proliferating cells than the MDA MB 231 (Figure 4). Treatment of MDA MB 231 cells with chemotherapy left the remaining cancer cells in a dormant state that could be restimulated to growth with LPS/EGF treatment and further treated with chemotherapy (Figure 5).

SA1 Task 2a (75% completed): Mouse model validation is on schedule despite a considerable delay in processing approvals. I have completed the mouse portion of the proposed optimization experiment with MDA MB 231. I am currently processing these tissues to determine optimal timepoints (6 months remaining on this task). On dissection of the mice I was able to ascertain that the mammary fatpad injections had been successful. Quantitative PCR of target organs and imaging are the current modalities underway to determine the metastatic timepoints needed for this year's larger experiment.

SA2 Tasks 1a and 2a (10% completed): Additionally I have received primary breast cancer specimens from 5 patients that I have processed and am optimizing culturing



Analytes	Fold increase
sEGFR	1.2
Follistatin	
Leptin	
Prolactin	
sVEGFR-1	
EGF	
IGFBP-1	
IL-18	
PAI-1	
uPA	1.7
IL-1b	
IL-1ra	
IL-6	
IL-7	1.3
IL-10	1.2
IL-12p70	1.3
IL-13	1.3
IL-15	1.3
IL-17A	1.4
Eotaxin	2.9
G-CSF	
GM-CSF	
IP-10	2.4
MCP-1	6.3
MIP-1a	1.5
PDGF-BB	
MIP-1b	1.4
RANTES	1.9
TNFA	
VEGF-A	1.8

Figure 6 Cell signaling analytes identified in preliminary metastasis screen by luminex technology. Supernatants from hepatic cultures on Day 15 with and without MDA MB 231 were compared (231/control) to determine fold increase in various signaling molecules in a luminex multiplex study. The BioRad Bio-Plex Pro Human 27-plex, Group I, Bio-Plex Pro Human Chemokine 40-plex, Bio-Plex Human Cancer 16-plex, and Bio-plex Human Cancer 18-plex panels were used. Resultant pg/mL data for each analyte was assessed for fold increase in metastatic conditions by dividing the conditions with cancer by those without. These candidate biomarkers can be tested moving forward in animal experiments and in human patient sera.

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

This has been accomplished both locally and nationally. I am part of the Pathology Post-doctoral Research Training Program (path.upmc.edu/PPRTP/index.htm) and the University of Pittsburgh Post-doctoral Association (www.uppda.pitt.edu). These University of Pittsburgh-based programs provide for mentorship and exposure to a multitude of career choices through programs such as Data and Dine, Women in Medicine and Science Forums, Postdoc to Postdoc, and regular workshops. In addition, regular seminars and meetings have also contributed to my training and include: the Magee Women’s Hospital weekly breast conference with clinicians and translational researchers, the Women’s Cancer Research Center weekly meetings, weekly

Department of Pathology seminars, and regular meetings with my mentors (Training plan Tasks 1a and b). Additionally laboratory weekly journal clubs and laboratory data meetings have provided important opportunities for presentation of my work.

Nationally, as a part of my training and professional development I have participated in four national conferences. I attended the American Association for Clinical Chemistry annual meeting (July 2014) where my poster was a finalist in the personalized medicine division. I attended this conference again in July of 2015 as well. I attended the American Society for Matrix Biology annual conference in October 2014 where my work was selected for oral presentation (Training plan Task 1c). I was also awarded the Paul E Strandjord Young Investigator Award (which covered some of my travel expenses) for the 50th Annual Academy of Clinical Laboratory Physicians and Scientists meeting in Minneapolis, MN in May of 2015. At this meeting I made an oral presentation of this research and competed against other trainees for the prestigious Young Investigator with Distinction Award – which I won. These meetings also provided key training seminars and networking opportunities.

For formalized training, in the summer of 2014 I attended an imaging course provided by the Center for Biologic Imaging at the University of Pittsburgh (Training Plan Task 2a). As demonstrated above I have also received training on the all human liver bioreactor as well as training in the detailed procedures for the mouse modeling sections (Training Plan Task 2b and c). Additionally, in August of 2015 I travelled to Boston to receive detailed training in Luminex studies and completed the study in Figure 6, which allowed me to probe the cytokine signals present in both my *in vitro* and *in vivo* model systems.

▪ **How were the results disseminated to communities of interest?**

Results were disseminated through national and local meetings (listed above) as well as biannual meetings of microphysiologic systems groups held by the NIH and NCATS in Washington DC (July 2014 and February 2015, August 2015).

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

This is my final report. I am in the process of writing additional publications, funded specifically under this award, an important part of my professional development. Additionally, I will be attending the San Antonio Breast Cancer Symposium 2015 in addition to at least one other national conference this year. To continue developing my training I will also be traveling to Boston to learn the Luminex technology which will allow me to probe the cytokine signals present in both my *in vitro* and *in vivo* model systems.

4. IMPACT:

a. **What was the impact on the development of the principal discipline(s) of the project?**

- i. In the field of metastatic breast cancer we have established the first all-human model of micrometastases that spontaneously undergo dormancy (Figures 1-6). This model is vital to begin to unravel the complex resistance seen with metastatic breast cancer, particularly the fear of recurrence 5-10 years after apparent cure. Using this model I am now probing the mechanisms of dormancy. This model also provides an ideal platform for the breast cancer community to perform preclinical studies on pharmacologic interventions in dormant or growing metastatic breast cancer.

b. **What was the impact on other disciplines?**

- i. This work also has implications for metastatic cancer generally as the liver is one of the major sites of metastases for solid tumors. This model, therefore, poises us to also evaluate interventions for other metastatic diseases.

c. **What was the impact on technology transfer?**

1. This model system has the potential to provide valuable preclinical information for pharmacologic companies, particularly in the area of cancer therapy. The liver is also the primary site of drug metabolism, thus this model links drug metabolism information with efficacy and toxicity.

d. **What was the impact on society beyond science and technology?**

1. *Nothing to report*

5. CHANGES/PROBLEMS:

a. **Changes in approach and reasons for change**

Nothing to report

b. **Actual or anticipated problems or delays and actions or plans to resolve them**

Nothing to report

c. **Changes that had a significant impact on expenditures**

Nothing to report

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

Nothing to report

e. **Significant changes in use or care of human subjects**

Nothing to report

f. **Significant changes in use or care of vertebrate animals.**

Nothing to report

g. **Significant changes in use of biohazards and/or select agents**

Nothing to report

6. PRODUCTS:

a. **Publications, conference papers, and presentations**

i. **Journal publications.**

Nothing to report

ii. **Books or other non-periodical, one-time publications.**

Nothing to report

b. **Other publications, conference papers, and presentations.**

Wheeler SE, Clark AM, Shepard Neiman J, Pillai VC, Venkataramanan R, Young CL, Lauffenburger D, Griffith L, Stolz DB, Wells A. Stiffness of the micro-environment modulates tumor dormancy and chemotherapy resistance in a hepatic all-human microphysiologic model of metastasis. The American Society for Matrix Biology October 12-15, 2014 (**oral presentation**)

Wheeler SE, Clark AM, Young CL, Pillai VC, Shepard J, Stolz DB, Venkataramanan R, Lauffenburger DA, Griffith LG, Wells A. Breast Cancer Metastasis: Dormancy and Re-emergence. 50th annual Academy of Clinical Laboratory Physicians and Scientists (ACLPS) meeting, Minneapolis, MN May 28-30, 2015. (**platform presentation**)

c. **Website(s) or other Internet site(s)**

Nothing to report

d. **Technologies or techniques**

Nothing to report

e. **Inventions, patent applications, and/or licenses**

Nothing to report

f. **Other Products**

- Paul E Strandjord Young Investigator Award. 50th annual Academy of Clinical Laboratory Physicians and Scientists (ACLPS) meeting, Minneapolis, MN.

- Young Investigator with Distinction Award for oral presentation. 50th annual Academy of Clinical Laboratory Physicians and Scientists (ACLPS) meeting, Minneapolis, MN. May 28-30, 2015.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

a. **What individuals have worked on the project?**

Name:	Sarah Wheeler
Project Role:	Post-doctoral fellow

Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	12
Contribution to Project:	Project PI, performed and designed all experiments.
Funding Support:	This award (BC130651)

b. **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Nothing to report

c. **What other organizations were involved as partners?**

1. **Organization Name:** Massachusetts Institute of Technology (MIT)
2. **Location of Organization:** Boston, MA
3. **Partner's contribution to the project**
 - a. **Collaboration** I receive mentorship from Dr. Linda Griffith and collaborate with the post-doctoral fellows in her laboratory on the all-human bioreactor system and hydrogel scaffolds.
4. **Organization Name:** Veterans Affairs Pittsburgh Healthcare System
5. **Location of Organization:** Pittsburgh, PA
6. **Partner's contribution to the project**
 - a. **In-kind support** The VA makes equipment and space available to me for a portion of this work.
 - b. **Facilities** Animal work is performed at the VA facilities.

8. SPECIAL REPORTING REQUIREMENTS

- a. **COLLABORATIVE AWARDS:**
- b. **QUAD CHARTS:**

9. APPENDICES: