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Award Number: W81XWH-10-1-1013

TITLE: Escape from Tumor Cell Dormancy

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REPORT DATE: October 2011

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
October 2011	Annual	22 September 2010 – 21 September 2011

4. TITLE AND SUBTITLE

Escape from Tumor Cell Dormancy

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-10-1-1013

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Massachusetts Institute of Technology
Cambridge, MA 02139

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

An insidiously terrifying aspect of breast cancer is its propensity to recur in metastatic sites even over a decade after all evidence of cancer has passed. It is obvious that these cells had escaped very early from the primary tumor as this occurs even in small, node-negative and in situ primary lesions, and that these micrometastases survival chemotherapeutic regimens that shrink and extirpate the primary carcinomas. Thus this mortal turn of events leads to three key questions – how do the cells escape early?, how do they survive over extended periods?, and what causes these dormant lesions to become aggressive at these late dates? The area of metastatic dissemination of primary cells has received a great level of inspection with an understanding of underlying molecular mechanisms, even if we do not yet have therapies. While chemotherapy survival in ectopic sites has been studies more recently, this is usually done in the context of a growing lesion. The truly under-appreciated and under-studied aspect is the last, that of re-emergence from dormancy. Understanding what triggers dormant breast cancer cells to emerge and form frank and mortal metastases would allow the development not only of rationale therapeutics but of prevention and possibly lifestyle avoidance. Herein, these issues are addressed using a novel organotypic bioreactor in which tumor cells can be followed for weeks to months, the process of seeding, dormancy and emergence can be followed.

15. SUBJECT TERMS
breast cancer, metastasis, tumor biology, novel models, tumor dormancy

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
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ESCAPE FROM TUMOR CELL DORMANCY

An Organotypic Liver System to Study Tumor Cell Dormancy

Alan Wells and Donna Stolz (UPitt), Linda Griffith (MIT)

INTRODUCTION: An insiduously terrifying aspect of breast cancer is its propensity to recur in metastatic sites even over a decade after all evidence of cancer has passed. It is obvious that these cells had escaped very early from the primary tumor as this occurs even in small, node-negative and in situ primary lesions, and that these micrometastases survival chemotherapeutic regimens that shrink and extirpate the primary carcinomas. Thus this mortal turn of events leads to three key questions – how do the cells escape early?, how do they survive over extended periods?, and what causes these dormant lesions to become aggressive at these late dates? The area of metastatic dissemination of primary cells has received a great level of inspection with an understanding of underlying molecular mechanisms, even if we do not yet have therapies. While chemotherapy survival in ectopic sites has been studies more recently, this is usually done in the context of a growing lesion. The truly under-appreciated and under-studied aspect is the last, that of re-emergence from dormancy. *Understanding what triggers dormant breast cancer cells to emerge and form frank and mortal metastases would allow the development not only of rationale therapeutics but of prevention and possibly lifestyle avoidance.*

The dearth of experimental insights into dormancy and the transition that heralds metastatic emergenceis due mainly to the lack of tractable experimental systems with which to probe this critical question. We proposed to use a novel ex vivo liver bioreactor to study this question. *We adapted this liver bioreactor to the study of metastatic competency in our original BCRP-funded work.* Metastases to the liver is one of the three main sites of metastasis and a major site for metastatic emergence after many years. We proposed that dormancy and emergence from it are linked together. Our model of metastatic seeding posited that the disseminated cancer cells undergo a reversion of the initial EMT, to re-establish E-cadherin-based connections in the distant soft organs. These E-cadherin adhesions would not only provide survival signals but also limit proliferation – the definition of dormancy. We proposed to take the next step and *hypothesized that the microenvironment surrounding the dormant micrometastasis is induced to produce growth factors and/or cytokines that downregulate E-cadherin, relieving the breast cancer cells from suppression.*

BODY: The accepted Statement of Work (Table 1) described a series of tasks to accomplish the two Objectives. We have tackled these Tasks in the order of greatest yield so that work in areas can progress as systems are being optimized in others. The main efforts during the first year of this two-year project have been focused on the establishing the system to be tested during the second year.

Table 1. Statement of Work

Work to be performed at University of Pittsburgh (Wells and Stolz Laboratories):

Objective 1:

1. isolate human hepatocytes and endothelial cells (months 1-24)
2. optimize protocols for isolation of human stellate and Kupffer cells (months 1-6)
3. isolate human stellate and Kupffer cells (months 7-24)
3. seed bioreactors with cells (months 1-24)
4. label tumor cells for fluorescence (months 1-6)
5. label tumor cells for mass reporting (months 3-9)

Objective 2:

1. generate liver organ bioreactors for tumor cell seeding (months 3-24)
2. seed organotypic liver bioreactors with tumor cells (months 3-24)

3. select and introduce inflammatory factors (months 9-24)
4. select and introduce stimuli to initiate 'inflammation' in situ (months 15-24)

Work to be performed at MIT (Griffith Laboratory):

Objective 1:

1. design bioreactor scaffolds (months 1-12)
2. optimize new high throughput bioreactor (months 1-12)
3. produce bioreactor scaffolds (months 1-24)

Objective 2:

1. produce bioreactors and scaffolds for utilization (months 1-24)
2. optimize the stiffness of the scaffolds (months 6-24)
3. optimize bioreactor sampling and input ports (months 9-24)

Work at University of Pittsburgh

Objective 1: The five tasks are proceeding apace. We have optimized the protocols for isolating the hepatocytes and non-parenchymal cells. The hepatocytes are routinely obtained mainly from human livers discarded as part of resections for colorectal metastases. We also have a protocol optimized for rat hepatocytes.

For the non-parenchymal cells, we have a protocol optimized to collect the entire fraction. This is good for both human and rat fractions. Separating the components is a current focus with an optimized protocol for both the large vessel and sinusoidal endothelial cells. The protocols for the Kupffer and stellate cells will be adapted to the tumor metastasis bioreactor from existing protocols for organotypic bioreactors.

The various breast carcinoma cells are being labeled by chemically and genetically. Cell tracker has been used to stain the cells so we can visualize membranes and shapes rapidly with minimal manipulations. This is useful for primary cells in that it is fast and does not require passaging. This will last for 7 to 14 days depending on the proliferation rate of the cells (as the label both leaches and gets distributed between daughter cells). For the cell lines, we have expressed various fluorescent proteins (mainly GFP and RFP). This labelling lasts for weeks and is maintained even after cell division as it derives from a CMV promoter-driven transcription. However, as the (G/R)FP requires cell transfection, selection and flow sorting, it is not appropriate for primary cell isolates with limited expansion potentials and the need for polyclonal representation.

Thus, the tasks for Objective 1 are largely completed or on the way to completion.

Objective 2: The tasks for Objective 2 are in progress. The soft-gel-matrix bioreactor scaffolds are being developed at MIT for integration with the bioreactor, and thus the work using this newer system at University of Pittsburgh has not yet initiated. An additional feature of the bioreactor has been developed (oxygen sensing) to improve monitoring of the physiological status of the cultures; as cells are stimulated by inflammation, or tumor cells escape from dormancy and proliferate, oxygen uptake increases.

The standard bioreactor with stiff-matrix scaffolds has been used to seed tumor cells and test for responsiveness to chemotherapeutic agents (see Chao et al, in revision). Even in the partially-activated environment of this stiff-sided bioreactor (where cells along the stiff wall are subject to activation), the presence of the microenvironment confers a differentiation-dependent partial resistance to a broad spectrum of anti-cancer agents. This differentiation state effect is a partial reversion towards a more epithelial phenotype (see Chao et al, Cancer Microenvironment); while E-cadherin is upregulated and makes cell-cell connections, vimentin and other mesenchymal markers remain expressed.

The main part of the work in Year 2 will focus on employing the new bioreactors with supports of extracellular matrix-like mechanical compliance to enhance physiological state,

along with oxygen sensing to measure cell physiology.

Work at MIT

The bioreactor format has traditionally used thin (0.2 mm thick) wafer-like scaffolds crafted from silicon, polycarbonate, or polystyrene by etching or drilling an array through-holes. The scaffold is then placed into the bioreactor on top of a 5um pore-size filter, thus creating an array of tiny wells into which cells are seeded. Cells attach to the walls of the through-holes but not to the filter, allowing the flow of medium through the cell mass to be reversed following the initial attachment period. Creating a soft gel scaffold format that is functional in all aspects (sturdy, stable, and physiologically relevant) is a multi-step process involving integration of desirable biological properties with desirable fabrication and mechanical properties. From the biology standpoint, cells must initially attach to the gel material in a biologically-relevant manner (i.e., one that does not promote excessive spreading or lack of cell-cell cohesion) and maintain attachment with minimal or modest remodeling of the gel, and the gel cannot globally degrade over the time course of the experiments although local remodeling may be desirable. From the fabrication and mechanical standpoint, a major challenge is the relative fragility of gels compared to stiff substrates, which affects both the handling properties as well as the sturdiness under flow forces. A preferred approach is thus to create gels that are either covalently bonded to the filter material surface or that penetrate into the filter during gelation and form physical links around filter structures, and to then support these filter-gel constructs with a frame that fits into the bioreactor.

We have created free-standing microarrays of PEG-fibrinogen gels that facilitate formation and maintenance of 3D hepatocellular tissue structures (Williams et al, 2011) and demonstrated maintenance of liver tissue function in these structures, where the entire scaffold was made from the hydrogel (i.e., the gels were not attached to a filter). A challenge in adapting these gels to the filter-bioreactor scaffold format is the substantial degree of swelling (>1.5X) these gels undergo following photopolymerization of the gel precursor solution. Swelling creates interfacial stresses that cause detachment of the gel from the filter or scaffold. We are addressing this by combining a previous observation that synthetic peptide gels functionalized with a dimeric RGD peptide, a heparin-binding domain from fibronectin, and EGF promote hepatocellular function (Mehta et al, 2010) with previous work using RGD-modified PEG gels of defined mechanical properties (Peyton et al, 2011) to create robust synthetic gels with defined adhesion functionalities. This is an iterative process to identify conditions that provide appropriate adhesion functionalities, mechanical properties, and low degree of swelling. A second challenge is to create micropatterns of hydrogels on the filter substrates. One constraint is that regions of the filter must remain gel-free, to allow the flow of culture medium that provides both nutrient distribution as well as mechanical stress. Molding techniques that work well with impermeable substrates are not very amenable to use with porous filters, as the macromers flow into all regions of the filter. Photolithography, an alternative to molding, can be challenging when the feature sizes of the gel structures are relatively tall (>250um). We are modifying both of these approaches iteratively with new gel precursor formulations to control swelling and stiffness on filter-polymerized micropatterned gels.

A significant constraint in bioreactor operation for analysis of escape from dormancy is controlling transport of oxygen, which is needed to feed the growing tumor. In homeostatic liver tissue in the bioreactor, a major constraint on reactor design is ensuring adequate renewal of oxygen depleted by respiring tissue. We tested 3 different designs to assess the effects of how oxygen transport is affected by the surface area of the air/liquid interface, and in turn, how the steady-state function of primary hepatocytes is affected by the steady-state oxygen concentration at the tissue inlet. Based on these experiments, we have determined a limit on minimum oxygen transfer and have a basis for building reactors for specific applications. Concomitant with these experiments, we developed a new method for measuring oxygen accurately and more

economically with ruthenium probes. Measurement of oxygen is a highly informative means of assessing the proliferation of cells in the bioreactor.

KEY RESEARCH ACCOMPLISHMENTS:

- Defined cell isolation protocols
- Labeled tumor cells for tracking
- Coordinated bioreactor design with cell seeding
- Defined design parameters for bioreactor specifications based on oxygen delivery for tumor cells "escaping from dormancy" in the context of homeostatic liver.
- Defined 3 approaches to creating soft hydrogel scaffolds.

REPORTABLE OUTCOMES:

Articles:

A Wells, YL Chao, J Grahovac, Q Wu, DA Lauffenburger (2011). Cell motility in carcinoma metastasis as modulated by switching between epithelial and mesenchymal phenotypes. Frontiers in Bioscience 16, 815-837. PMID: 21196205.

YL Chao, Q Wu, M Acquafondata, R Dhir, **A Wells** (2011). Partial mesenchymal to epithelial reverting transition in breast and prostate cancer metastases. Cancer Microenvironment, in press.

YL Chao, Q Wu, C Shepard, **A Wells** (2011). Hepatocyte-induced re-expression of E-cadherin in breast and prostate cancer cells increases chemoresistance. in revision.

CM, Williams, G. Mehta, S.R. Peyton, A.S. Zeiger, K.J Van Vliet, **L.G Griffith** (2011) Autocrine-controlled formation and function of tissue-like aggregates by primary hepatocytes in micropatterned hydrogel arrays, Tissue Engineering, Part A 17:1055-68 PMID: 21121876

Abstracts:

D Taylor, **A Wells** (2011). Priming the metastatic niche: A role for stress-induced breast cancer dormancy? Department of Pathology Annual Retreat, Pittsburgh, PA.

D Taylor, **L Griffith, A Wells** (2011). Breast cancer in the metastatic niche: A role for stress-induced dormancy and emergence? Era of Hope Meeting, Orlando, FL.

L Griffith (2010). Breast Cancer Drug Development: The Patient/Scientist View. Merrimack Pharmaceuticals, Cambridge, MA.

CONCLUSION:

The first year has been successful in setting the stage for development of a bioreactor that will not spontaneously activate the inflammatory state. This will enable more human-like seeding and tracking of the breast cancer cells in the target organ, with direct manipulation of the cytokine milieu. We are poised for completion of the study in the second year of the program.

The knowledge gained in the first year is only now being prepared for dissemination. Still we have learned, and will report on, the implications of mechanical stiffness on organ milieu and adaptability of tumor and stromal cells. This represents a fundamental change in the way one must view, and thus design, ex vivo bioreactors to truly capture the human condition.

Recommended changes:

None