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PRINCIPAL INVESTIGATOR: Gregory Hannon

CONTRACTING ORGANIZATION: Cold Spring Harbor Laboratory
Cold Spring Harbor, NY 11724

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14. ABSTRACT The purpose of this collaborative project is to establish a molecular definition of the dormant state of a cancer cell. In doing this we hope to understand how this dormancy is broken, ultimately leading to recurrence in a patient that was stably in remission. Once our understanding of this is more complete it is hoped that we can devise strategies for secondary preventions. This funding year we have successfully optimized the two-photon microscopy for the single cells from a tissue section and have established how to use the current technology to extract RNA and make high quality transcriptome libraries from low input materials. We anticipate additional advances in the quality of the transcriptome libraries from a single cell, fixed in PFA, as new technology is anticipated to be released before the end of the year. We have also made progress on strategies for identifying dormant cells in human biopsies.						
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Introduction

The primary purpose of our collaborative research project is to discover the genes and pathways that regulate breast cancer dormancy. This information will allow us to generate hypotheses about the mechanisms underlying dormancy maintenance as well as how it is broken. We will use models of breast cancer wherein murine or human breast cancer cells establish and progress through dormancy. We will utilize innovative methods to perform laser capture microdissection of cancer cells in the dormant state as well as cancer cells that are progressing from dormancy. These cells, which are at distinct stages of cancer progression within their native environments, will be molecularly profiled. We will also profile various cell-types within the micro-environment that contact cancer cells. This molecular description of the dormant state will allow us to perform loss-of-function and gain-of-function studies of candidate drivers and suppressors of dormancy progression. Ultimately, we anticipate the identification of genes that could serve as attractive targets of therapeutic inhibition. While the Tavazoie lab has been working towards human breast cancer profiling, The Hannon lab has been optimizing conditions for identifying and profiling dormant cells. In our first and second year of funding we have been working towards two goals. One is the ability to locate dormant cells and the second is the ability to capture and profile, using RNA seq, single cells.

Body

Subtask 1a.

In task 1, we had proposed to characterize the molecular features of dormant cells by identifying these cells in metastatic micro-environments through the use of automated serial two-photon tomography (STPT).

The Hannon lab has been working towards this goal by developing and optimizing the methodology required for this.

1. We have established the necessary techniques for perfusion, fixation and agarose embedding of mouse tissue prior to processing with the Two-Photon Microscope (TPM). We have optimized a number of cellular injection protocols to enable tumor metastatic cells to be detected in various organs within the mouse, specifically, fatpad injections, with and without primary tumor removal (training sought from the Egeblad lab at CSHL), intravenous injections (tumor cells primarily delivered to the lungs), mesenteric vein injections (tumor cells sent directly to the liver, but with metastatic cells also delivered to the lungs), and cardiac injections. The cardiac injection is a far less invasive technique compared to the mesenteric procedure and minimizes first-pass filtration through the pulmonary capillaries (lungs), allowing the maximal number of metastatic breast cancer cells to reach the bone. Mice undergoing this procedure will be analyzed at two time points: 1-2 weeks post injection to evaluate lung and liver metastasis and 4-6 weeks post injection to evaluate bone metastasis.

We have successfully combined GFP-H2B (nuclear) labeled mouse breast tumor cells from the 4T1 cell line with Isolectin GS-IB4, Alexa Fluor 568 conjugate, to visualize both the tumor landscape and the blood vessel distribution, Fig.1A, and as shown in Fig.1B we are now able to clearly identify small micromets and single cells within a mouse organ.

A

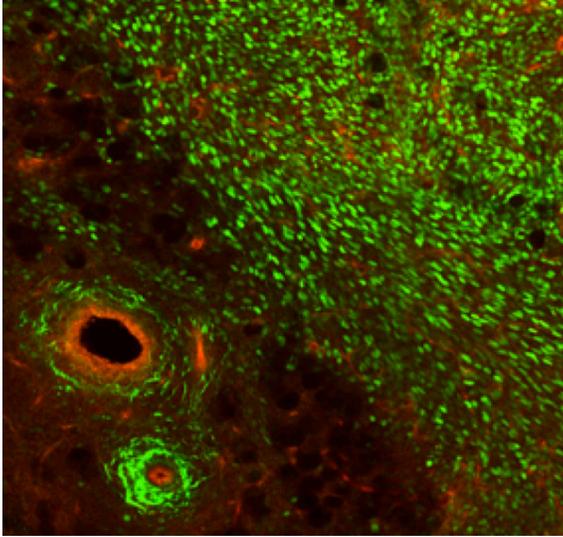


Fig.1A. Two photon microscopy of a primary tumor (green (GFP-H2B)), and interspersed vessels stained with Isolectin (red).

B

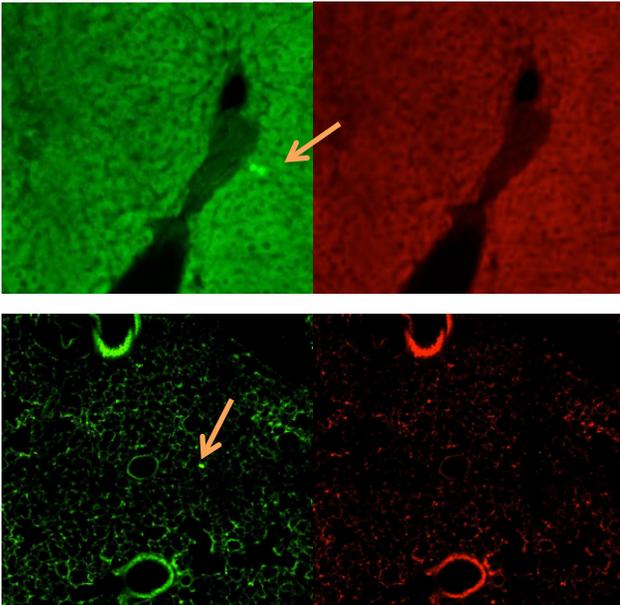


Fig.1B. Two photon microscopy of (Top) 2 GFP-H2B 4T1 cells within the liver and (Lower) a single cell in the lung. Both the red and green channels are shown to illustrate above background signal.

2. This funding year we identified some problems with the GFP labeled cells and their ability to metastasis. This is necessary, as despite the use of cardiac injections to simulate metastases, to truly identify cells that remain dormant in the most natural setting we need cells that have left the primary tumour. Our experiments identified that the H2B-GFP cells were creating an immune response restricting their ability to disperse and grow away from the primary tumor. We have been creating and testing the use of alternate labeling tags, CFP, mCHERRY, and venus, in the hope that these will produce less of an immune response. In addition we have been optimizing virus infections testing a range of virus promoter that would be better suited for 4t1 and human cells. We have identified good better promoters that ones we are currently using and will be working towards switching the optimized florescent tags into the new plasmids.
3. This year we have been working on creating BACS for micro-environment cell and, cell status, specific markers enable us to create mice tagged for these cell types. We have met with some challenges with the methodology for BAC recombineering, but have thus far confirmed the development of two BACs, one for CD2 (myeloid cells) and one for HIF2 (and cancer associated gene). We are working on a number of others e.g. lymphoid and endothelial, and other cancer related genes.
4. Having established the feasibility of locating sparsely distributed single tumor cells within an organ using the TPM, we are now optimizing the techniques to extract the single cell. This is met with some challenges that we are working towards over coming. The first being, how to identify and select the tissue section containing the single cell from the hundreds of tissue sections that are collected post imaging. Two ideas are being worked on. The simplest is using a mix of both GFP (for florescent imaging) and Trypan blue (for bright field) stained cells in high concentrations and inserted at angles through the agarose, adjacent to the tissue, so that the distance between the two insertions will provide some indication of section position relative to the top and bottom of the tissue, as illustrated in Fig. 2. The second is a purpose built collection device that will collect tissue sections in the order that they are sliced off the block in dishes with approx. 10 slices per dish. This collection machine is currently under construction but a machine similar to our design is already in use by one other lab and we plan to meet with the lab early in the following year.

In anticipation of using one of these methods we have been optimizing the technique best suitable for extracting the cell in order to extract RNA. This year and last year we have been working on two techniques for this. The first is with the use of a laser capture microscope (LCM). The tissue sections from the TPM are 50uM in depth, therefore in order for the tissue to be suitable for the LCM we have been working on the paraffin embedding technique to enable further sectioning from 50uM to 10uM. One hurdle we will overcome here is the loss of GFP signal upon paraffin embedding. We are currently working on methods to quickly stain, with immunofluorescence (IF), the cell of interest to allow capture by LCM. Alternatively we are working on methods of tissue enzymatic disaggregation to separate the cell of interest from other cells. Using a combination of Dispase and Collagenase Ia and mechanical passing through a 35 mkm mesh we are able to create a single cell suspension and locate GFP positive tumor cells from an area of liver tissue, as shown in Fig. 3

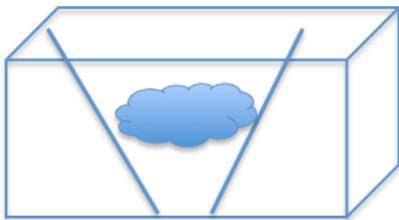


Fig.2. An illustration of how a tube of cells imbedded within the agarose, at angles, will denote a relative position of a tissue section that can be correlated with imagery data.

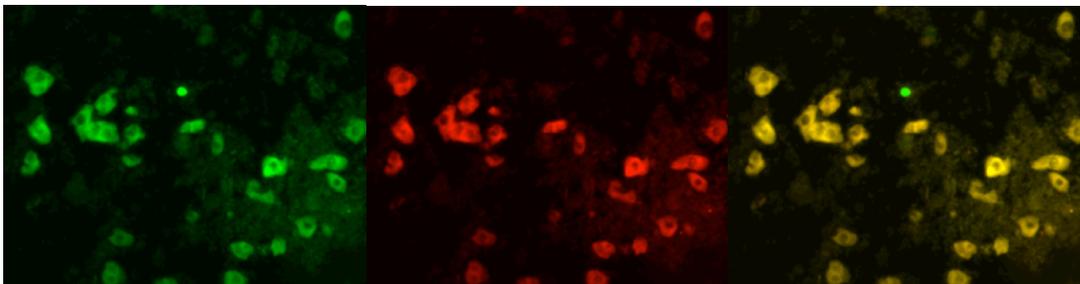


Fig.3. Single cell suspension of a tissue section, post TPM, after enzymatic digestion for 3 hours and mechanical disruption through a mesh. left. GFP channel, middle, Red channel to indicate background and right, the merging of the GFP and RED channels.

- During this funding year we have also been working on the use of EdU labeled cells to aid in identification and recovery of dormant cells. Labeled cells retain EdU signal when they are not dividing (dividing cells dilute the signal and therefore appear less bright). This enables us to identify potentially dormant cells, and also allows us to harvest these cells via FAC sorting methods. Work on this technique is establishing which of our current methods work well with the EdU protocols and we have some initial data suggesting the feasibility of identifying EdU positive cells in an established tumor and in a distant organ (Fig. 4)

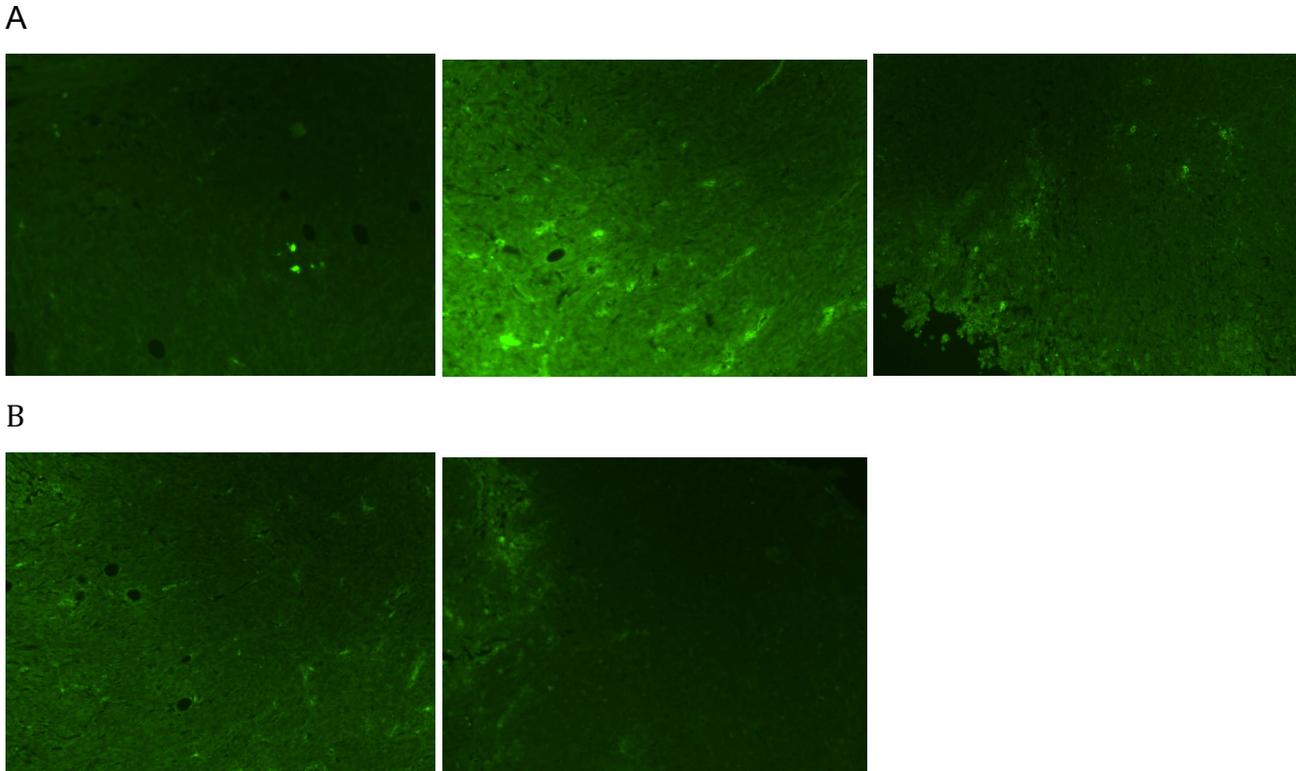


Figure 4. A) an established tumour of EdU 4T1 cells, stained post death with secondary click chemistry. The far left figure identifies two possible dormant cells. B) the same tumour as A) but without the secondary click chemistry.

- We have also been establishing RNA seq methods for single cell sequencing from both fresh and frozen cells. For this we have been using flow cytometry (FACs) to isolate single 4T1 cells and groups of 10 and 100 4T1 cells, and compared this with low input pure RNA from the same cell line. We have tested a number of kits with and without bead purification, namely the Clontech #634823 "SMARTer® Ultra□ Low Input RNA for Illumina® Sequencing - HV"; the NuGen #7102 "Ovation® RNA-Seq System V2" - for cDNA synthesis, the Clontech #634947 "Low Input Library Prep Kit"; the NuGen #0330 "Ovation Ultralow DR Multiplex System 1-8" -

for library preparation, and we are currently first in line to receive the Nugen single cell RNA seq kit. So far we have established very high % mapping (>90%) with strong correlation between 10 frozen cells and 100 frozen cells, and produced high quality libraries with input as low as 10pg using the clonetech reactions. The PFA fixed cells produce approx. 50% mapping with 10 cells last year, now methods have improved and this funding year has enabled a mapping percentage of approximately 80%.

Subtask 1b.

In this subtask, we had proposed to interrogate human biopsy samples for dormant tumor cells and determine if the expression levels of genes identified to be over-expressed in the transition from dormant to micro-metastases stratify patients into those that relapse and those that do not. Although we have not yet established a gene expression list for clinical validation analyses, we are working on combining the techniques we are establishing in mice with more human tissue specific techniques. We have searched the literature and found two proteins thought to specifically label human breast cells, with higher expression in breast tumor cells, these being Mammaglobin and MUCL1. We have tested their expression using immunohistochemistry and QPCR, in 6 human cell lines and for controls, 2 pancreatic cell lines and 2 prostate cell lines. We are also developing whole mount staining techniques to stain mouse organs containing human breast cells that can then be visualized using TPM, with the intention that we will locate human dormant breast cancer cells in human biopsies. This year we have been working on the development of a single chain antibody for mammaglobin, using alpacas. It is hoped that the reduced size for the single chain antibody will allow whole mount and slide staining to work far more efficiently and allow us to fluorescently tag the antibody, thereby reducing immunostaining times and improving RNA recovery.

Subtask 2b.

In this subtask, we had proposed to functionally test the roles of genes identified as being differentially expressed in micro-metastatic tumor cells relative to dormant cells through loss-of-function and gain-of-function analyses. In order to initiate these studies, we first had to characterize and optimize the human breast cancer in vivo dormancy systems that will be subsequently used for the profiling methods. In the coming year, we anticipate the identification of genes differentially expressed by these cancer cell populations and their micro-environments and will proceed to functional testing of these genes.

Subtask 3a.

In this subtask, we had proposed to modulate, through over-expression or knockdown, genes that are predicted to maintain dormancy cell survival. This subtask will be pursued in future years of the funding period upon identification of differentially expressed genes that are candidate dormancy regulators.

Subtask 3b.

As per above, This subtask will be pursued in future years of the funding period upon identification of differentially expressed genes that are candidate dormancy regulators.

Subtask 3c.

As per above, This subtask will be pursued in future years of the funding period upon identification of differentially expressed genes that are candidate dormancy regulators.

Subtask 3b.

As per above, This subtask will be pursued in future years of the funding period upon identification of differentially expressed genes that are candidate dormancy regulators.

Key Research Accomplishments to date from the Hannon lab

1. Establishing ideal cell transplantation methods for achieving single dormant cells.
2. Establishing the methodology for Two Photon imaging of tissue, and the ability to identify lone single cells.
3. Improving the quality RNA seq libraries from low input RNA and comparisons with library construction kits between fixed and frozen tissue.
4. Continued training of a Research Technician in the Hannon lab in tissue culture, animal handling and molecular techniques.
5. Improving viral infections and reducing immune responses for the florescent proteins.
6. Developing a single chain antibody for mammaglobin.
7. Designing and developing florescent cell specific BACs

Reportable Outcomes

1. Manuscripts

Nothing to report at this time

2. Presentations

Nothing to report at this time

3. Funding applied for based on work supported by this award

Current reporting period:

4. Research/training opportunities

This grant has directly supported postdoctoral training for Drs. Elena Rozhkova and Clare Rebbeck. In addition, the full training of one research assistant, Meagan Keane, who recently graduated from the University of Maryland.

5. Employment Opportunities

At various times over the life of the project, this grant has funded the full-time employment of two postdoctoral associates and one research assistants.

6. Databases

Nothing to report at this time

Conclusion

In summary, the Hannon lab has optimized and established solid methodology for Two Photon microscopy of dormant cells. We have designed and tested methods for extracting these single cells from a tissue section and gained significant advances in our ability to construct RNA seq libraries from low input and PFA damaged RNA, above the previous year. All these methodologies will be combined with the cancer cell line work carried out by the Tavazoie lab in order to obtain candidate genes for dormancy. We have initiated and part established the necessary BAC constructs for analyzing the micro-environment surrounding a dormant cell, and we have been working on two new methods for identifying dormant cells, for human, the development of a single chain antibody to mammaglobin, and for mice the use of EdU, as well as improving the current methods.