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TITLE: Understanding Tumor Dormancy as a Means of Secondary Prevention

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CONTRACTING ORGANIZATION: Cancer Research UK, London, GB

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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 prevention. This funding year we have pursued an alternative strategy for the identification of dormant tumor cells and the characterization of their microenvironment: Imaging mass cytometry or IMC. Over the past year, we have constructed two

15. SUBJECT TERMS

Breast cancer, Dormancy, metastasis, EMT, RNA seq, heterogeneity, ASNS

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1. INTRODUCTION

The primary purpose of our collaborative research project is to discover the genes and pathways that regulate breast cancer dormancy. Our plan is to use models of breast cancer wherein murine or human breast cancer cells establish and progress through dormancy and 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, will be molecularly profiled along with various cell-types within the micro- environment that contact cancer cells. This molecular description of the dormant state will eventually 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 the last four years 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.

2. KEYWORDS

Breast cancer, Dormancy, metastasis, EMT, RNA seq, heterogeneity, ASNS

3. ACCOMPLISHMENTS

Task 1 – Broadly to identify dormant tumor cells and characterize these and their environment

We continue to pursue means to identify dormant cells. As it was already mentioned in our last report, we have been significantly hampered by the extended period of time that it has taken to complete the purchase of the STPT microscope. This microscope is finally operational and, as expected, it is completed with a collection device capable of collecting 25 micron slices and automatically transferring them to microscope slides with a >95% capture rate. During the last year we have been spending considerable amount of time optimizing the embedding, the sectioning and collection of slices from different samples, including breast tumor. We are now able to section and collect automatically and reliably 25 micron sections and we are working on reducing the thickness to 15 micron, which is more amenable for some of the downstream processing. One example of a 4T1 tumor sectioned and imaged by us on the STPT is shown in Fig.1. We are now ready to start imaging and sectioning some of our dormancy models, such as lungs of mice bearing 4T1 metastatic breast cancer tumors.

After STPT sectioning of distant sites, the next step will be distinguishing dormant cells from cells that are progressing from dormancy as one of the main objectives of this project is to identify these different states of cancer cells and microdissect them separately to perform RNA seq analysis. An alternative approach will be to isolate single cells or nuclei enzymatically to perform single cell transcriptomics analysis using the 10x Genomics technology, which we already have available on site. It has also been recently established that is feasible to isolate cells or nuclei from frozen tissue or sections and use them to preform single cell RNA sequencing using this technology. Either one of the methods (microdissection or single cell sequencing), will allow us to reveal the characteristic molecular profile of a dormant cell as well as what changes are observed in this profile when tumor cells exit dormancy. The best way to define a dormant cell is the ability to remain at a distant site, without proliferating, until the dormant state is somehow broken. It therefore follows that one of the best ways to distinguish cells at different stages during the progression through dormancy, will be to follow the behavior of tumor cells seeded at distant sites over time. This requires the direct

visualization of isolated tumor cells through non-invasive methods in live animals, which was so far practically impossible. This has changed with the introduction of a more sensitive form of luciferase, the Akaluc, which was recently engineered by random mutagenesis from the firefly luciferase (Iwano et al. 2018. *Science* 359: 935-939). The Akaluc is able to convert a modified substrate to produce a bio-luminescent signal in the near infrared spectrum, which is visible through substantial depths of tissues, therefore allowing for the detection of single cells deep inside animals. The Akaluc can be used to visualize even single tumor cells in the lungs of mice (as shown in the publication), making it sensitive enough to allow the visualization of dormant cells via non-invasive bioluminescence imaging.

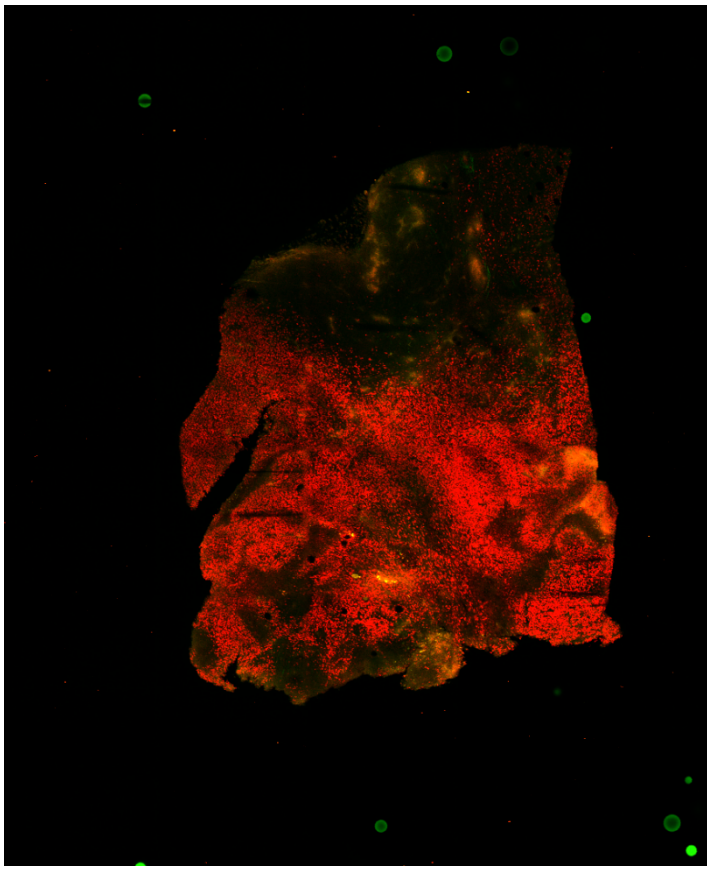
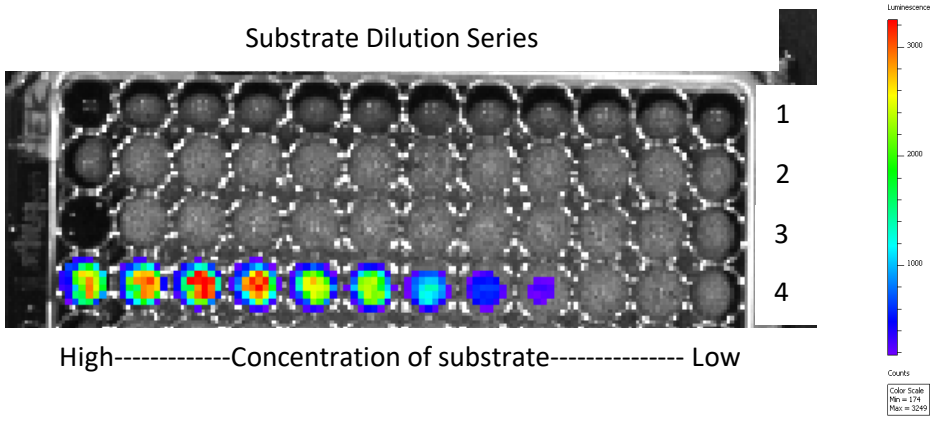


FIG.1 – Reconstruction of 4T1 breast tumor section using the STPT

4T1 cells were infected with a m-cherry reporter (red) and orthotopically injected in the mammary gland of Balb/c mice to form tumors. Represented is the reconstruction of a section of a 4T1 tumor following STPT imaging and sectioning. Individual tumor cells are visible in the red channel. GFP-labeled beads (green spheres outside the tissue) were embedded together with the sample as reference to realign the STPT images with subsequent images taken after further processing of the STPS sections, for example by IMC.

We are therefore planning to use the Akaluc to follow over time the behavior of tumor cells seeded at distant sites in order to identify cells at different stages of the progression through dormancy. We will then proceed to tissue collection and retrieval of the cells of interest by STPT sectioning, followed by laser capture microdissection to perform the molecular analysis of the tumor cells at different stages and the surrounding microenvironment. This approach should allow us to distinguish cells that have just maintained their number over time (bona-fide dormant cells), as opposed to the ones that proliferated or might have recently arrived from a primary tumor or developing metastasis. We have produced viral constructs containing an Akaluc-IRES-GFP cassette and used them to infected 4T1 tumor cells (Fig. 2). These cells therefore express both Akaluc and GFP and they can be tracked both by bioluminescence and fluorescence microscopy. We are now orthotopically injecting these cells to form mammary tumors and we will follow them over time at distant sites using the Akaluc bioluminescence. Once dormant and metastatic cells can be observed at distant sites, we will extract the tissue and section it using the STPT using the GFP reporter to find the Akaluc

positive cells and microdissect them using laser capture. We anticipate that this approach will allow for the isolation of bona-fide dormant cells, cells that recently exited dormancy and metastatic cells, as well as their surrounding microenvironment. This will eventually allow us to perform their molecular characterization and to compare between the different cell states, hopefully revealing the molecular profiles that are associated with the dormant state and the progression through dormancy.



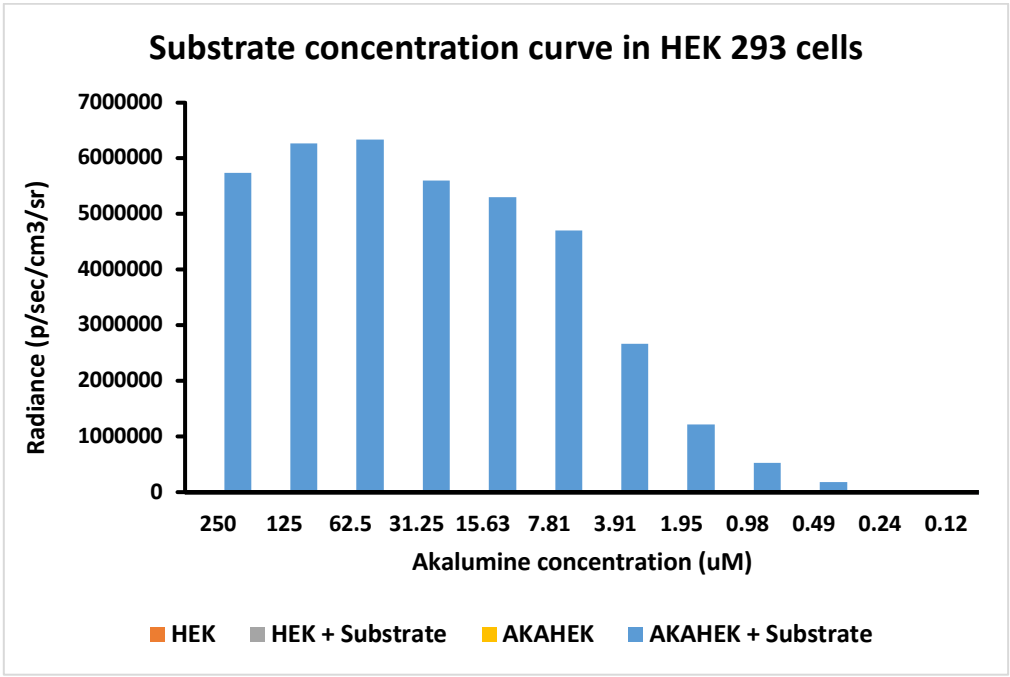


FIG. 2 –Akaluc bioluminescence in HEK 293 cells

Top: HEK 293 cells were transfected with Akaluc containing plasmid or left untransfected as control. Luminescence was measured at the IVIS in the absence or presence of substrate (1: HEK only, 2: HEK + substrate, 3: HEK + AKALUC, 4: HEK + AKALUC + substrate).
Bottom: Same samples analysed at the Pherastar. Radiance of control samples without Akaluc or without substrate is too low to be visible in the graph.

As mentioned in the last report we also pursued the alternative approach of using Imaging Mass Cytometry (IMC) to characterize both the status of breast tumor cells, as well as the immune infiltrates interacting with them. We had already optimized and validated two different panels of antibodies that are designed to characterize either the tumor cells or the immune infiltrate. In the last year we have acquired the Hyperion™ Imaging System and started optimizing its use on tumor tissue, even following sectioning on the STPT (Fig. 3). We are now geared to start using it on our own dormancy models or on the human tissue samples within the collaboration with Alana Welm and Cyrus Ghajar.

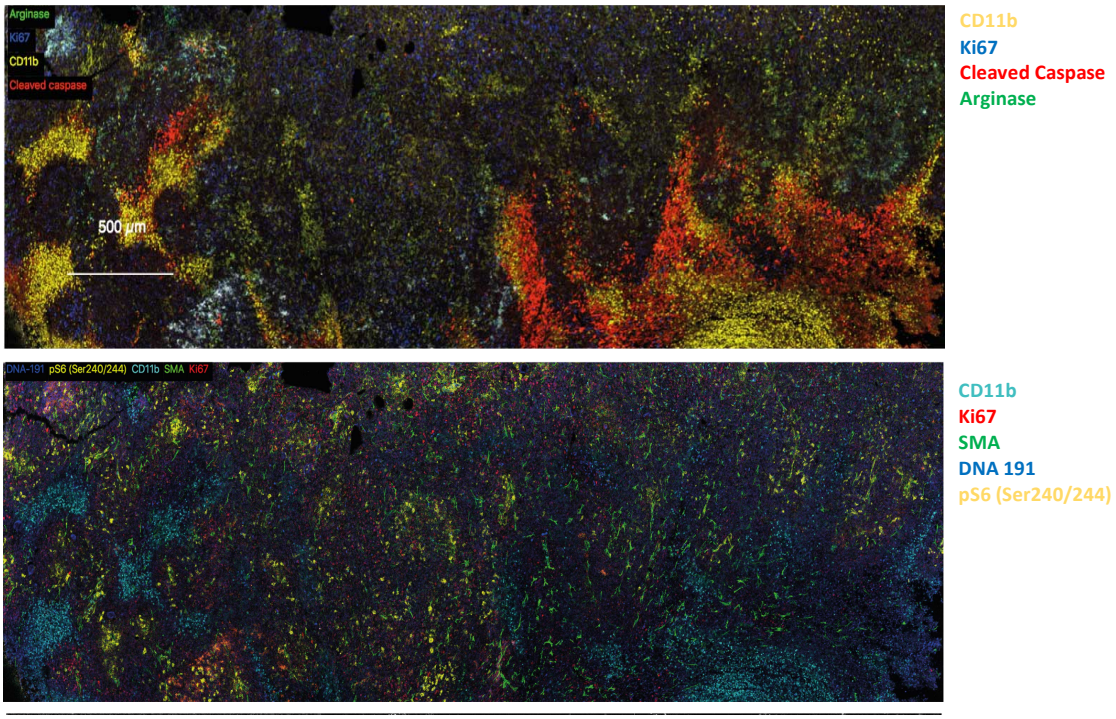


FIG. 3 – Imaging Mass Cytometry on a 4T1 breast tumor sectioned with the STPT

Example of IMC run on one 15 micron section collected from a 4T1 tumor after STPT sectioning. The two panels represent the same section showing the distribution of some of the antibodies used during the run. Note the cleaved caspase positive areas surrounding the $CD11b^{+}$ infiltrate (top), CD11b and Ki67 are shown in both panels as a reference.

We are also making progress in developing an artificial library of llama nanobodies that we could then screen for any target of interest to produce antibodies for targets that are not yet included in our current panels. Nanobodies will be particularly suited for the use in IMC because they are expected to be able to retain their specificity even after the conjugation process with metals, which is a necessary step for their use in any technique involving mass cytometry.

Tasks 2 and 3 – to identify candidate dormancy regulators and find ways to manipulate these for patient benefit.

In last year report have discussed in detail the use of the 4T1 model of breast cancer to identify possible drivers of metastasis in mice. We performed all these experiments using NSG immunocompromised mice. Given that tumor microenvironment and immune system are major components influencing both metastatic potential and metastatic dormancy, we are now expanding our previous studies on 4T1 cells to examine their behavior in recipient mice with

different immunocompetency. In our original experiments, we only considered NSG mice but we are now repeating the same experiments comparing NSG with Balb/c and nude mice. The Balb/c mice represent the fully immunocompetent end of the spectrum, whereas the NSG mice lie at the other end completely lacking the adaptive immune system and having defects in the innate immune response. The nude mice represent an intermediate stage being immunocompromised, but still retaining functional dendritic cells and macrophages which are instead absent in NSG mice.

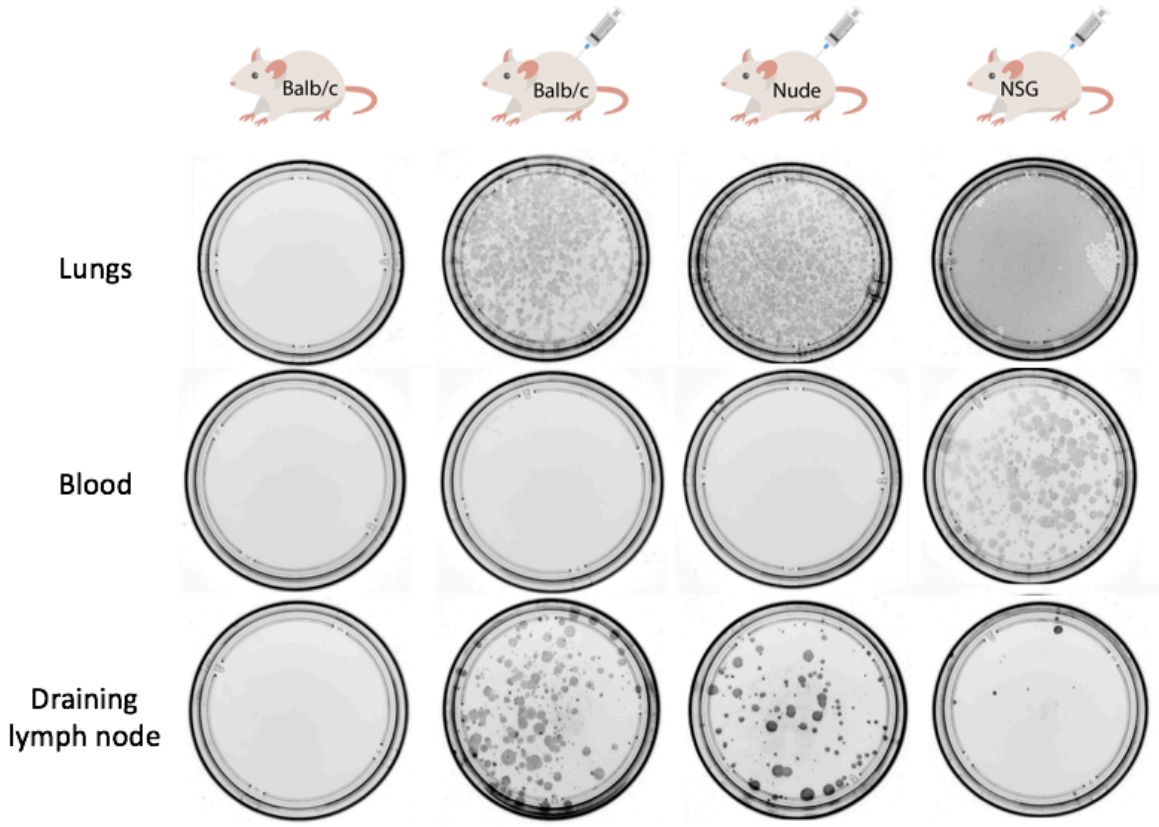


FIG. 4 – Metastatic potential of 4T1 tumors in different strains of mice.

4T1 cells were orthotopically injected in the mammary gland of three mouse strains with different immunocompetency to produce primary tumors. After 25 days potential secondary sites were collected and put in culture in the presence of 6-thioguanine. Since 4T1 cells are resistant to 6-thioguanine, they will form colonies that can be counted to estimate their metastatic potential in the different secondary sites. Note that metastasis to the lungs occurs in all mice, with the highest amount in NSG, where the plate is completely full. Interestingly, the route of dissemination seems different among different strains with the blood being preferred in NSG mice and the lymphatic system instead prevalent in the progressively more immunocompetent Nude and Balb/c strains.

Our preliminary results suggest that in mice with a higher degree of immunocompetency (Balb/c and Balb/c nude), 4T1 cells tend to metastasize preferentially through the lymphatic system as they are readily found in lymph nodes, whereas in NSG mice they seem to spread prevalently through the bloodstream (Fig. 4). In the next few months we will be looking into the relevance of this observation and try to establish whether lympho-angiogenesis and lymphatic system can play a role in mammary tumor dissemination in an immunocompetent setting, that more closely reflects the situation present in cancer patients.

In the last report we also mentioned that our studies on 4T1 tumors led to the identification of Asparagine Synthetase (Asns) as a driver of metastasis in NSG mice, strongly implicating asparagine bioavailability as a regulator of metastatic progression. This is also likely relevant in human cancers, as high ASNS expression is a marker of poor prognosis for many tumor types. One mechanism underlying our findings is likely a link between asparagine bioavailability and EMT, which can be observed both *in vitro* and *in vivo* (Knott et al. 2018. Nature 554:378-381).

Following up on this work, we are establishing a mass-spectrometry based assay to measure the ASNS activity in cells. We are making good progress on this and it will be soon ready to compare the levels of ASNS in different cells (for example the different clones of 4T1 described in our previous report) and check if it is sensitive and robust enough to measure changes in ASNS activity in different conditions. If this will be the case, we are hoping to be able to use this assay in the future to screen for ASNS inhibitors that could possibly be used as a mean to reduce the ability of breast cancer cells to metastasize and perhaps also influence their ability to enter or exit the dormant state.

A vast literature has associated EMT with resistance to therapeutic intervention. This encompasses cytotoxic chemotherapies as well as targeted therapies. Recent studies have even suggested that cells that have undergone EMT can evade immunotherapy. For a cell to remain dormant and serve as a seed of later disease it must evade a patient's initial treatment. The work described above has led us to the hypothesis that asparagine bioavailability can influence the potential of cells colonizing secondary sites. In high asparagine conditions, cells can adopt a mesenchymal phenotype and potentially occupy the perivascular niches that have been previously proposed as a site for DTC preservation. Restriction of asparagine through any of a number of mechanisms may negate this potential in the colonizing cell but might also reverse EMT later at the dormant niche and in micrometastases, revealing these cells to the immune system or to therapeutic intervention. Thus, we feel that this work represents a substantive step forward toward the overall goals of this grant.

4. IMPACT

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

This work has supported the training of a number of individuals. Several technicians have been trained as a part of this program. One of them has recently started her graduate studies on this same project. Clare Rebbeck has been promoted to Senior Research Associate during her time as part of this award. A postdoc, Ashley Nicholls, is acquiring new skills in drug discovery as we search of an ASNS inhibitor and another postdoc, Claire Mulvey, is acquiring new skills optimizing the STPT embedding and sectioning.

How were the reports disseminated to communities of interest?

Knott et al. (2018). Nature 554:378-381

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

For the remaining term of the grant, we wish to use the combination of bioluminescence (Akaluc) to follow the behavior of tumor cells at metastatic sites over time and the STPT to recover these cells after tissue collection. This will allow to identify and profile dormant tumor cells, generating candidate that, in the future, can be taken into gain and loss of function

studies to hopefully identify potential therapeutic targets. We will also characterize by IMC the microenvironments, with respect to immune cells, of dormant tumor cells, metastases, and micromets in several tissues from mouse and human. Finally, we hope to pursue our hypothesis that manipulating asparagine bioavailability would at very least improve initial therapy to reduce the pool of cells available to adopt the dormant state and at best, make DTCs vulnerable to either endogenous immune attack or therapeutic intervention.

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

Nothing to report

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

The observation that asparagine availability impacts metastasis could provide strategies for improving primary therapy and for reducing relapse.

5. CHANGES/PROBLEMS

The problems we have had acquiring the necessary equipment have impacted both the timeline and the trajectory of our work in this grant. Despite the fact that this grant is unfortunately almost run out, we will continue after the end of the grant to study tumor dormancy. The fact that we could not proceed with the project as we originally planned, provide the impetus to attempt our alternative approach that has provided very interesting and potentially important new directions.

6. PRODUCTS

Knott et al. (2018). Nature 554:378-381.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project (reporting period 09/30/17 – 9/30/18)?

Name: Clare Rebbeck
Project Role: Senior Research Associate (Post Doc)
Nearest person month worked: 3 months
Contribution to project: Project design, animal work, cell culture & imaging

Name: Ashley Nicholls
Project Role: Research Associate (Post Doc)
Nearest person month worked: 12 months
Contribution to project: Project design, animal work, cell culture & imaging

Name: Aarthi Gopinathan
Project Role: Research Associate (Post Doc)
Nearest person month worked: 6 months
Contribution to project: Animal work, cell culture & imaging

Name: Claire Mulvey
Project Role: Research Associate (Post Doc)
Nearest person month worked: 8 months
Contribution to project: STPT optimisation, cell culture & imaging

Name: Natasha Narayanan
Project Role: Student
Nearest person month worked: 12 months
Contribution to project: Animal work, cell culture & imaging

Name: Cristina Jauset Gonzalez
Project Role: Graduate Student
Nearest person month worked: 11 months
Contribution to project: Animal work, cell culture & imaging

Name: Sophia Wild
Project Role: Graduate Student
Nearest person month worked: 11 months
Contribution to project: Animal work, cell culture & imaging

Name: Tatjana Kovacevic
Project Role: Graduate Student
Nearest person month worked: 12 months
Contribution to project: Animal work, cell culture & imaging

Name: Fiona Nugent
Project Role: Research Assistant (technician)
Nearest person month worked: 7 months
Contribution to project: Animal work, cell culture & imaging

Name: Jose' Franco Alvarez
Project Role: Research Assistant (technician)
Nearest person month worked: 2 months
Contribution to project: Animal work, cell culture & imaging

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

The following grant has been awarded:

RP\EA\180015. Research Professorship Enhancement Award 2017, Royal Society
Project title: SYNTHETIC VHH LIBRARIES FOR CELLULAR STRUCTURAL BIOLOGY

The following grant has terminated:

R37 GM062534-16 (Hannon)
NIH/NCI

Roles of small RNAs in guarding germ cell genomes
The major goal of this project is: To understand the mechanistic basis of dsRNA-induced gene silencing using a biochemical approach.
Role: Primary Investigator

For complete details, please refer to Other Support documentation in the Appendix.

What other organizations were involved as partners?

N/A

8. APPENDICES

Greg Hannon Other Support

HANNON, GREG

OVERLAP
None

Cold Spring Harbor Laboratory
ACTIVE

63180501 (Hannon)	01/01/16 – 9/30/19	0.00 calendar*
PEW Charitable Trust	$ 617,349	
Glaucoma Research		

The major goal of this project is: to carry out research to understand further the biology of retinal ganglion loss. Role: Primary Investigator

OVERLAP
None

Cancer Research UK Cambridge Institute
ACTIVE

W81XWH-12-1-0300 (Hannon)	9/15/12 – 11/14/18	1.2 calendar
Rockefeller Univ PI Tavazoie	$ 1,284,058	
DOD Collaborative Scholars Award		

Understanding tumor dormancy as a means for secondary prevention
The major goal of this project is: to characterize the molecular and cellular basis of breast cancer dormancy and dormancy progression.
Role: Co-Primary Investigator

5 U01 MH106035-01 (Hannon) 9/26/14 – 6/30/19 1.2 calendar
NIH $ 267,600
An Optogenetic Toolkit for the interrogation and control of a single cell
The major goal of this project is: to develop a broad optogenetic toolkit, based on covalent protein tags and photoreleasable compounds, enabling the recovery of genetic material, the alteration of gene expression, and the insertion of transgenes to any cell of the brain with high spatial precision.
Role: Primary Investigator

W81XWH-14-1-0110 (Hannon) 9/30/14 – 9/29/19 0.6 calendar
DOD Breakthrough Award $ 1,711,170
The major goal of this project is: to molecularly characterize DCIS
Role: Primary Investigator

0028354 (Hannon) 4/1/15 – 9/30/18 1.2 calendar
PEW Charitable Trust $ 215,259
The major goal of this project is: to develop state of the art RNAi tools
Role: Primary Investigator

110161/Z/15/Z (Hannon) 8/1/16 – 7/31/23 1.2 calendar
Wellcome Trust Investigator Award £ 455,794
This is scholarship support for work on non-coding RNAs, including piRNAs and lncRNAs.
Role: Primary Investigator

C9545/A24042 5/1/17 – 4/30/23 1.2 calendar
CRUK Grand Challenge Award – IMAXT £ 2,408,121
The major goal of this project is: to image and molecularly annotate xenografts and tumors.
Role: Lead Co-Investigator

C14303/A17197 10/1/14 – 10/1/19 0.6 calendar
CRUK Core Award £ 655,888
The major goals of this project are: to understand the biological functions and mechanisms of non-coding RNAs; to attempt to tackle critical problems in cancer biology, with a major focus on breast and pancreatic cancer, to understand aspects of disease ranging from the genetics of early, non-invasive cancers to critical pathways driving metastasis; to develop new technologies in several areas including mining information inherent in tumor heterogeneity, optimization of CRISPR-based strategies, and building general platforms for control of biological processes by light.
Role: Primary Investigator

RP\EA\180015 10/01/17 - 03/31/21 1.2 calendar
Royal Society £ 58,333
Research Professorship Enhancement Award
SYNTHETIC VHH LIBRARIES FOR CELLULAR STRUCTURAL BIOLOGY
Role: Primary Investigator

OVERLAP
None

*Please note that no designated level of professional support is required by the funding organization for these projects, however, Dr Hannon has 3.6 calendar months of effort currently available at his discretion to apply to the oversight of these research endeavors and any other research or administrative responsibilities. At no time will his effort exceed 12 person months in any one year.