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14. ABSTRACT

The purpose of this project is to investigate molecular events occurring in the preclinical stages of mammary cancer. Specifically, the project investigates the intersection between the development of genome demethylation, retrotransposon transcriptional activity, and retrotransposon-driven transcription of cellular genes in an engineered mouse model of mammary cancer. After accomplishing all the preliminary activity proposed in the Statement of Work, the project has completed with slight delay the mouse breeding for the project's Year One, and progress has been made in collecting material for planned molecular analyses. A series of technical and logistic problems delayed completing planned work on the molecular aspect of the project. However the problems have been solved and we are in a position to move forward with the molecular analyses within the next month, We believe that the eventual findings will provide insights into understanding the role of genome hypomethylation and expression of retrotransposons in cancer ontogeny, and may impact cancer prevention in the future.

15. SUBJECT TERMS
Breast cancer, epigenetic, DNA methylation, retrotransposon, preclinical cancer, development, deep sequencing.

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Introduction

This project is designed to address the subject of mammary cancer development. The purpose of the project is to investigate molecular events occurring in the preclinical stages of mammary cancer; the results may lead to insights into cancer prevention in the future. Specifically, the project investigates the intersection between genome demethylation, retrotransposon transcriptional activity, and retrotransposon-driven transcription of cellular genes. Retrotransposon promoters are well recognized to function as alternative promoters for different cellular genes, generating chimeric transcripts that may or may not function in the same way as transcripts from the regular gene promoter. Transcriptional activation of retrotransposons is strongly linked with their CpG DNA methylation, and global genomic demethylation is one of the commonest molecular changes in malignancies. The project tests the hypothesis that, in preclinical stages of tumour development, progressive genomic demethylation leads to increased transcriptional activity of retrotransposons and this, in turn, leads to transcription of otherwise silent genes, potentially setting up molecular conditions that favour cancer development. We developed a genetically engineered mouse model in which a specific mammary cell population is fluorescently marked upon initial transcriptional activation of the SV40 large T antigen (SV40Tag) oncogene. SV40Tag is transcriptionally activated during pregnancy and lactation, and the mice are predisposed to develop mammary cancer after 3 pregnancies and lactations. Using this model, populations of marked cells can be collected for integrated analysis of gene expression, promoter usage, and DNA methylation after defined amounts of exposure to SV40Tag during different stages of preclinical cancer development.

Body

The principal roles of Dr. Peaston as a PI in this project are to establish mouse breeding and production colonies for the project, to collect and process mammary samples from the mice, forward samples for analysis to her collaborating colleagues at Washington University in St Louis and The Jackson Laboratory, and perform experiments testing analysis results.

Since initial proposal of the project, Dr. Peaston has moved from a full-time research scientist position at The Jackson Laboratory, Maine, USA, to an academic appointment in the new School of Animal and Veterinary Sciences (SAVS) at The University of Adelaide in Australia. In her current appointment, she has teaching, administration and clinical (veterinary) roles as well as continuing her basic research. SAVS is still establishing its academic programs, with the first students expected to graduate at the end of calendar year 2013. As a result of startup staffing difficulties, Dr Peaston has acquired additional unanticipated responsibilities. These include curriculum and lecture programme development and course coordination, teaching on more topics than originally expected, and assisting with clinical facility operating process development and implementation. Despite difficulties this has created with demands on her time, and other unanticipated technical difficulties outlined below, Dr Peaston has substantially completed the mouse production planned for the first year of the project, and has made progress in collecting and processing mammary samples from the mice. The following 5 tables and associated comments summarize progress in the original Statement of Work.

Table 1. Year 1 Preliminary Activity prior to start of funding

GOAL	Result
1. • Hire Research Assistant (RA) • Train RA	• Hiring delayed by difficulties negotiating university administrative requirements and structures. • Mr Timothy Smith hired, start date August 29 2011 • Training was completed over the first 6 months on the job. Training included mouse husbandry and colony management, mammary cell isolations in preparation for flow cytometry, DNA and RNA isolation from mouse tissue and cells, mouse genotyping. In addition, training included University-mandated induction courses.
2, 3. • Import stock mice 04/11 • establish breeding colonies	• Mice arrived 2nd May 2011 • Initial breeding colonies set up 2nd May 2011.

4. Double homozygous mTmG;WapCre males mated with C57BL6/J females	• Matings established on July 21 2011. The offspring of these matings were earmarked for pilot FACS experiments and then the matings discontinued
5. Starter colonies established	• Started expanding the mTmG;WapCre colony • initial matings of Waptag1 mice x C57BL6/J mice established July 21 2011. The purpose of this starter colony cross is to generate mice heterozygous for the SV40 oncogene, which are subsequently used in the production colony to cross with homozygous mTmG;WapCre mice, producing female offspring heterozygous mTmG;WapCre, and either SV40 null (control) or SV40 heterozygous (tumour prone).
6. Travel	• June 9-10 2011. Dr Peaston presented a seminar on Mammary Gland Development in a Mammary Stem Cell workshop at The Jackson Laboratory with the sponsorship of StemCell Technologies Inc. • June 13 2011 Dr Peaston met with Dr. Carol Bult, Dr Hibbs unavailable. Agreed to postpone regular e-meetings until material and data were being exchanged with The Jackson Laboratory. • June 14 2011 Dr Peaston met with Dr. Edwards' lab group at Washinton University in St Louis, and presented a seminar on the project. Agreed to informal e-meetings until project and material transfer to Wash U commenced.

Table 2. Months 1-3 (Sept-Nov)

GOAL	Result
1. Establish mTmG;WapCre x heterozygous Waptag1 production colonies	• Completed setup of initial production colony trios as planned. • 10 matings established October 19. • 11 matings established November 22. 11 matings were of heterozygous Waptag1 males x homozygous mTmG;WapCre females, 10 were of the reciprocal cross.
2. Collect mammary epithelial cells from virgin mTmG;WapCre and mTmG;WapCre;Waptag1 mice and from tumours if available, extract RNA/DNA	• Not completed because of problems arising with the flow cytometry facility (see note below this table)
3. Ship materials	Not done (see above)
4. Schedule for e-meetings meetings	Communications established as in 6.Travel in Preliminary Activity above.

Notes on Table 2.

1. The Laboratory Animal housing and flow cytometry facility are located adjacent to one another, however Dr. Peaston's laboratory space is located over 1 hour away by car. To overcome logistical problems arising, Dr. Peaston arranged to process mouse mammary tissue using bench space in a colleague's lab located in the same building as the flow cytometry facility.
2. pilot FACS experiments in early to mid December with mTmG;WapCre mice gave conflicting results and much smaller numbers of cells were recovered than expected from extensive prior work at The Jackson Laboratory. Discussion with the FACS technical officer was unproductive in determining what was the problem.
3. RNA and DNA were not isolated from the small number of cells collected as it was not clear that this FACS procedure was yielding the appropriate cells. The cells were discarded.
4. The remainder of female triple transgenic mice generated during this time were therefore allocated to experimental matings until a solution for the FACS problem could be found.

Table 3. Months 4-6 (Dec-Feb)

GOAL	Results
1a. set up Replicate #1 triparous experimental females	• Completed as follows • 26 Dec 2011 26 females in trio matings (2 females per male) • 10 Jan 2012 24 females in trio matings • 24 Jan 2012 23 females in duo or trio matings (1 female per male) • 07 Feb 2012 27 females in trio or duo matings
1b. Set up Replicate #1 experimental uniparous females, 2 cohorts	• Completed with minor delay • 22 Feb 2012 22 females in duo matings • 06 Mar 2012 31 females in duo and a few trio matings Analysis of litter production by trio matings set up for the first two cohorts of triparous mice showed 23% to 33% failed to become pregnant over two weeks. Therefore further stud males were trained starting in mid-January to allow duo matings. The number of stud males therefore expanded to and remains at 28 males dedicated to stud duty for each new cohort of females. In addition, 21 stud males have been added to the colony for staggered mating as required to produce triparous females.
2. Mammary tumour cell collection	• One WapTag1 mouse developed a mammary tumour during this period but FACS problems were unresolved and the tumour was not collected.
3-9.	• Unable to be completed – while FACS problems were unresolved, all triple transgenic mice were allocated to uniparous or triparous cohorts, and work was concentrated in this area since it was deemed that mouse production and matings were the most time-consuming activity for the project and had first priority.

Notes on Table 3. In addition to the problems outlined above, the project encountered three additional problems in this quarter.

1. Production colony trios were expected to produce between 4 and 6 litters averaging 6 pups each to produce roughly 60 pups every 3 weeks to generate at least 28 experimental female mice on average. In December, analysis of the production colony matings showed that mTmG;WapCre females x Waptag1 heterozygous males weaned fewer pups over 4 – 6 litters than the reciprocal crosses. This appeared associated with reduced numbers weaned in the 2nd and 3rd litters, and approximately 25% of the trios failed to produce more than 2 litters before being culled for poor reproductive performance. Starting in late December, these matings were gradually replaced by matings using WapTag1 heterozygous females x homozygous mTmG;WapCre males, and pup production improved and stabilized at the expected level (average 6 pups per litter over 4 litters per trio).
2. In addition, in January the institution introduced a compulsory major change in mouse housing arrangements from open top cages to individually ventilated cages with about half the floor area of the previous cages. This represented an improvement in conditions in the long term, but we believe it may have contributed to the dip in productivity, and required re-negotiating cage charges with the facility to match the original budgeted cage charges.
3. Analysis of litter production by trio matings set up for the first two cohorts of triparous experimental mice showed 23% to 33% failed to become pregnant over two weeks. In an attempt to improve this, further stud males were trained starting in mid-January to allow duo matings. The number of stud males therefore expanded to and remains at 28 males dedicated to stud duty for each new cohort of females. In addition, 21 stud males have been added to the colony for staggered mating as required to produce triparous females. While these measures have not improved the pregnancy rate of virgin mice, they have made tracking production per mouse simpler. The current pregnancy rate for virgin mice (replicates 1 and 2 of uniparous and triparous females) averages 68% (range 46-86). In the triparous mice, the pregnancy rate for mating 2 is 81.5% (data so far from replicates 1 and 2) and for mating 3 is 95% (summary data so far from replicate 1 only).

Table 4. Months 7 - 9 (Mar - May)

GOAL	Results

1a. Set up Replicate #1 experimental uniparous females, 2 cohorts	• Completed • 20 Mar 2012 30 females in duo matings • 03 April 2012 31 females in duo matings
1b. Set up Replicate #2 experimental triparous females, 4 cohorts	• Completed with modest delay • 17 Apr 2012 27 females in duo matings • 22 May 2012 28 females in duo matings • 05 Jun 2012 29 females in duo matings • 26 Jun 2012 30 females in duo matings At the beginning of this quarter the experimental mouse breeding was about 2 weeks behind the planned schedule. In late April we introduced a further 2-week delay while finalizing alternative arrangements for FAC sorting cells in order to ease the pressure on number of cages in the mouseroom.
2. Replicate #1 uniparous mice harvest cells, isolate RNA/DNA	• Cells were collected from 37 control and 30 tumour-prone mice from this replicate. • Collection of cells was started in late May and completed by late July • RNA/DNA extraction from frozen cells was started in August and will be complete by the beginning of October; Dr Peaston's lab tech responsible for this took 4 weeks annual leave from late August to late September.
3-5	• Not completed since DNA and RNA not yet shipped
Supplementary: collection of tumour and virgin mammary cells	• Pending from previous quarters • No tumours have become available since the flow cytometry arrangement was settled • Triple transgenic control and tumour-prone females still allocated to experimental cohorts as a first priority.

Notes on Table 4.

- It should be noted that during this quarter, in late April, a satisfactory alternative arrangement for FAC sorting cells was established within the original facility, but working with different personnel and machinery.
- At this time, it became clear that the prior arrangement with Dr Peaston's colleague to use lab bench space for tissue processing was unsatisfactory due to time restrictions placed on Dr Peaston's access to the lab as a result of increased student numbers in the lab.
- A new and more satisfactory arrangement for bench space sharing was made with an academic colleague on the University of Adelaide campus a short walk from the flow cytometry facility.
- Pilot experiments in May satisfactorily demonstrated sorting and collection of green fluorescent cells from the background of red fluorescent cells, allowing collection of uniparous replicate one to proceed.

Table 5. Months 10 - 12 (Jun - Aug)

GOAL	Results
1a. Set up Replicate #2 experimental uniparous females, 4 cohorts	• Completed with modest delay • 07 Jul 2012 26 females in duo matings • 24 Jul 2012 28 females in duo matings • 07 Aug 2012 28 females in duo matings • 21 Aug 2012 29 females in duo matings
1b. Set up Replicate #3 experimental triparous females, 4 cohorts	• Postponed to September 2012; started 9/11/2012
2a. Replicate 1 triparous 4 cohorts harvest cells, isolate RNA/DNA	• Postponed to the next quarter
2b. replicate 2 uniparous	• Postponed to the next quarter

harvest cells and isolate RNA/DNA	
3-8	• Not done because previous requirements not yet met
Additional note 1.	As the experimental cohorts near completion, triple transgenic virgin control and tumor-prone mice are now (September) being set aside for mammary tissue collection as envisaged for Months 1-3. Collection of mammary tissue from them is planned to be completed by mid-november 2012 and RNA/DNA shipped in December.
Additional note 2	Our collaborator Dr Hibbs recently left The Jackson Laboratory. Dr. Bult, our other collaborator at The Jackson Laboratory, has assured Dr. Peaston that she will take his place in final data analysis. With her continuing involvement, RNA samples can be processed as originally planned, and preliminary analyses performed within the Scientific Services group at The Jackson Laboratory.

Key Research Accomplishments

The project has made substantial progress in several respects:

- Establishing mouse production and experimental colonies under fluid circumstances
- Replicates 1 and 2 of uniparous and triparous mice are either complete or nearly complete, the third replicates will be completed by March or early April 2013.
- Establishing a workable sample pipeline in Adelaide
- Preliminary quantification and qc of RNA and DNA aliquots from Replicate #1 uniparous mice confirm that the number of mice killed for mammary cell extraction are sufficient to yield the quantity of RNA and DNA required for the planned analyses, particularly in the light of technical advances made by our collaborator Dr John Edwards.

Reportable Outcomes

- Seminar “Retrotransposons and mammary cancer” The Basil Hetzel Institute, Queen Elizabeth Hospital, Adelaide. November 23, 2011.

Conclusion

The project has suffered a number of important technical and logistic difficulties particularly in regard to FACS performance and laboratory access close to the FACS facility that were thought to have been solved before the project started. These have significantly delayed all the planned molecular analyses. However, with mouse production substantially accomplished, and the first RNA/DNA isolated, we are poised to move ahead quickly with molecular aspects of the project. In particular, the delayed methylome analyses will benefit from Dr. Edwards improved and streamlined Methyl-MAPS protocol and analysis pipeline. We believe that two and probably all three experimental replicates can be analysed as planned and substantially verified in the time remaining in the project.