

Award Number: W81XWH-05-1-0506

TITLE: Breast Stem Cell Markers and Tumor Stem Cells in BRCA1, BRCA2 and Non-BRCA 1/2 Women

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REPORT DATE: August 2006

TYPE OF REPORT: Final

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

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE 01-08-2006		2. REPORT TYPE Final		3. DATES COVERED 31 Jul 2005– 30 Jul 2006	
4. TITLE AND SUBTITLE Breast Stem Cell Markers and Tumor Stem Cells in BRCA1, BRCA2 and Non-BRCA 1/2 Women				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-05-1-0506	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Geoffrey J Lindeman, Ph.D., Jane E Visvade, Ph.D., Joseph Sambrook, Ph.D., François Vaillant, Ph.D., and Natasha Forrest Email: lindeman@wehi.edu.au				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) The Walter & Eliza Hall Institute Melbourne 3050 Australia				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 Original contains colored plates: ALL DTIC reproductions will be in black and white.					
14. ABSTRACT It is believed that certain breast tumors originate in either a breast stem or progenitor cell. Notably, tumors that arise in women carrying a BRCA1 gene mutation often exhibit a 'basal' phenotype that may reflect their origin in the breast stem cell. We therefore hypothesized that the breast stem cell pool is aberrant in breast tissue of BRCA1 (or BRCA2) carriers versus non-carriers and that it becomes progressively and distinctively expanded in older carriers. To evaluate this concept, we performed a pilot study in which we obtained archival samples from breast tissue from the Kathleen Cuninghame Foundation Consortium for Research into Familial Breast Cancer (kConFab). We first derived and analysed subpopulations of breast tissue for the expression of putative stem cell markers and investigated means to derive short-term in vitro cultures. Our preliminary findings indicate that it is possible to identify distinct subpopulations in normal breast tissue and tissue derived from BRCA mutations carriers. These studies are now being extended to study putative stem and progenitor populations using reduction mammoplasties and prophylactic mastectomy specimens.					
15. SUBJECT TERMS Breast stem cells, BRCA1, Basal subtype, breast cancer					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	11	19b. TELEPHONE NUMBER (include area code)

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INTRODUCTION

Preliminary work by our group and others (1,2) has led to the prospective isolation of mammary stem cells in mice. Interestingly, the mouse mammary stem cell-enriched population expresses basal markers and is negative for the steroid receptors ER and PR, as well as ErbB2 expression. This phenotype is reminiscent of the ‘basal subtype’ of breast cancer (3). This observation lends support of the notion that basal tumors are derived from aberrant stem cells. The phenotype of the human breast stem cell has yet to be established. Nevertheless, certain markers have been identified that could enable the prospective fractionation of breast tissue (including ESA, MUC, CD44, CD24 and the capacity to efflux Hoechst dye), and it seems likely that a subset of cells selected using these or other markers will be enriched for breast stem cells.

Increasingly, certain breast tumors are believed to originate in either a breast stem or progenitor cell. Since tumors that arise in women carrying a BRCA1 gene mutation exhibit a ‘basal’ phenotype, we hypothesized that the breast stem cell pool is aberrant in breast tissue of BRCA1 (and possibly BRCA2) carriers versus non-carriers and that it becomes progressively and distinctively expanded in older carriers. The aims of our study were to evaluate stem cell characteristics in breast tissue derived from either BRCA1 or BRCA2 carriers, women with a strong family history where no mutation had been identified (nonBRCA1/2 women) and to compare these with breast tissue from normal women.

BODY

To evaluate the concept that the stem cell pool is perturbed in BRCA carriers, we performed a pilot study in which we obtained archival samples from breast tissue from the Kathleen Cuninghame Foundation Consortium for Research into Familial Breast Cancer (kConFab). This consortium has accumulated over 1000 families with a strong family history of breast cancer and collects blood samples as well as tissue at the time of surgery (with patient consent), including prophylactic mastectomies. The proposed tasks for this project included:

1. Evaluation of putative breast epithelial stem cell markers
2. Derivation of short-term cultures
3. Establishment of tumor xenograft models using NOD/SCID mice

We first derived and analysed subpopulations of breast tissue for the expression of putative stem cell markers and investigated means to derive short-term *in vitro* cultures. Our preliminary findings indicate that it is indeed possible to identify distinct subpopulations in normal breast tissue and tissue derived from BRCA1 and BRCA2 mutation carriers and a representative example is shown in Figure 1. Cell suspensions were stained with a variety of cell surface markers. Dead cells were excluded on the basis of propidium iodide staining (PI) and further evaluation made on the PI low fraction (PI box in Figure 1). Next, haematopoietic and endothelial cells were excluded on the basis of CD45 and CD31 expression and lineage negative cells selected (third plot in Figure 1). Preliminary findings suggest that it is possible to fractionate the Lineage minus population on the basis of several markers. Two representative Markers, A and B are shown in the bottom plot in Figure 1.

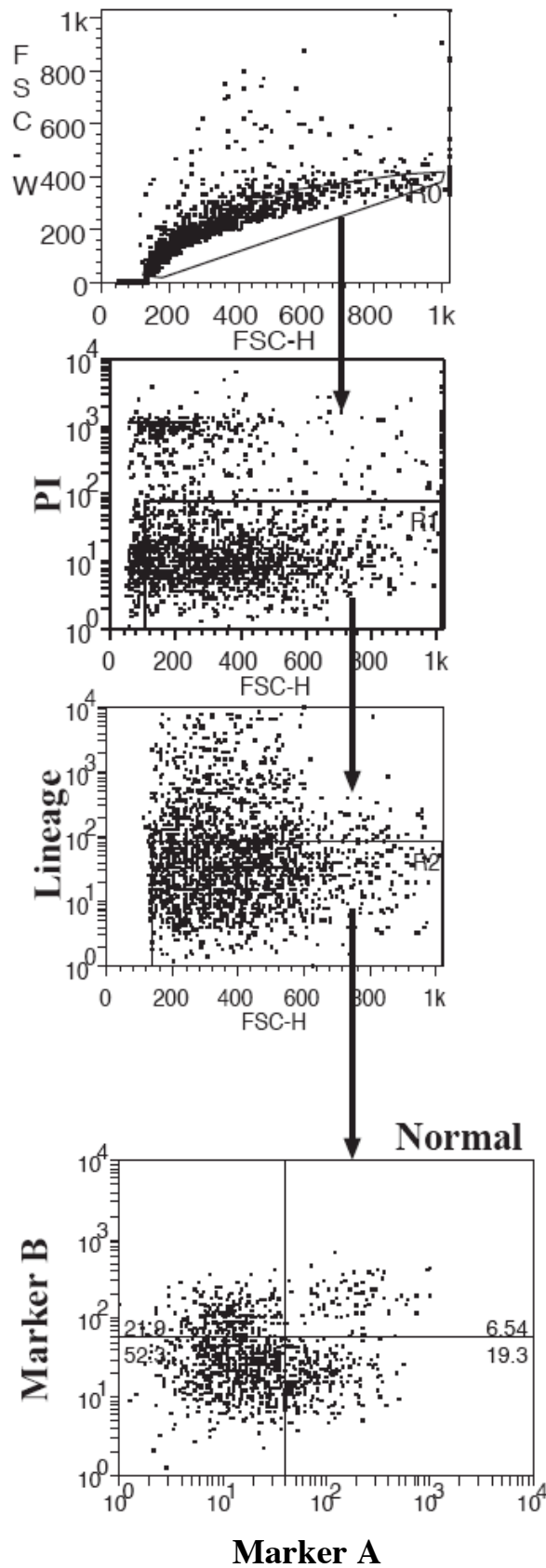


Figure 1. Fractionation of human breast tissue by Flow Cytometric Analysis.

These findings made during the period of DOD Concept grant support our now being extended to study putative stem and progenitor populations using reduction

mammoplasties and prophylactic mastectomy specimens, as we have since found that freshly prepared tissue is superior to samples that have been frozen in DMSO.

Short-term cultures have also been generated using archival material and a modified *in vitro* assay as previously described using mouse mammary epithelial cells (1). Our study has revealed that different subpopulations generate colonies with variable efficiency, suggesting that some may be enriched for stem/progenitor capacity. (Figure 2).

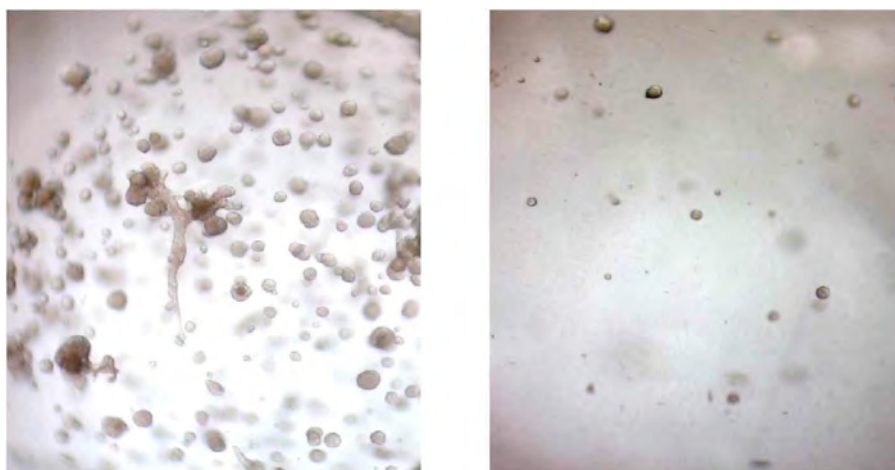


Figure 2. Derivation of short-term *in vitro* cultures. Subpopulations of cells were cultured in Matrigel using modified protocol as described by Shackleton et al (1). In the pilot experiment shown, one subpopulation gave rise to numerous heterogeneous colonies (left panel). The right panel is a representative image of a subpopulation that gave rise to fewer colonies.

Insufficient sample numbers of suitable quality were available to determine whether there are qualitative differences between BRCA1, BRCA2, nonBRCA1/2 and control samples. However, this work will now be extended in future studies using prospectively collected tissue samples.

During the course of the DOD Grant funded period, we were able to establish the humanized NOD/SCID xenograft model in our laboratory. As ‘proof of principle’ mammary outgrowths were successfully derived using unsorted cell suspensions. One such outgrowth is shown in Figure 3. This work has placed us in a good position to prospectively collect fresh breast tissue and transplant sorted samples into recipient mice.

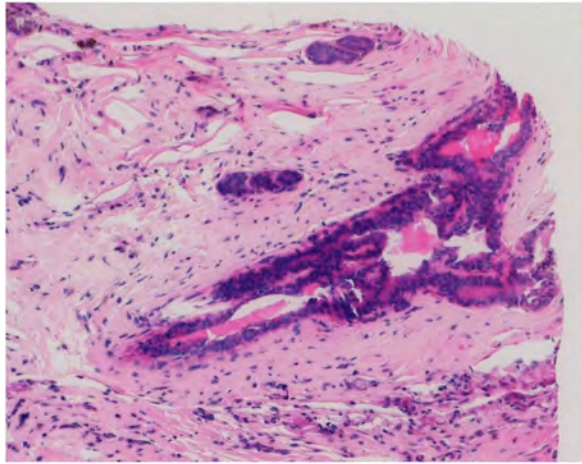


Figure 3. Histological section of a humanised mouse mammary fat pad. The fat pad, which was cleared of epithelial elements at puberty, contains an outgrowth that has arise from human epithelial cells.

KEY RESEARCH ACCOMPLISHMENTS

- Establishment of a short-term *in vitro* culture system to assay subpopulations of breast tissue
- Establishment of the humanized NOD/SCID xenograft model to grow human breast tissue
- Identification of cell surface markers that define breast epithelial subpopulations

REPORTABLE OUTCOMES

Publications:

Pending – the concept award served as a ‘pilot’ project to establish model systems for the study of breast epithelial populations using archival material. We are now in a position to proceed with a more comprehensive project aimed at prospectively defining breast epithelial subpopulations using fresh breast tissue and to determine whether these are altered in women who harbor BRCA1 and BRCA2 mutations. This is necessarily a long-term project and the US DOD grant support will be recognised in future publication(s).

Abstracts and presentations:

Geoffrey Lindeman:

- 28th Annual Meeting, ANZ Breast Cancer Trials Group, Cairns, Queensland, Australia, July 2006 (Speaker)
Title: How can we Improve the Breast Cancer Treatment Paradigm?
Author(s): Lindeman GJ
Abstract: none
- Familial Cancer – Research and Practice 2006, Annual Scientific Meeting, Couran Cove, Queensland, August 2006 (Speaker)
Title: Negative hormone receptor status of mouse mammary stem cells reminiscent of human basal breast tumours
Author(s): Asselin-Labat M-L, Shackleton M, Stingl J, Vaillant F, Forrest NC, Eaves CJ, Visvader JE, Lindeman GJ
Abstract: refer appendix

Jane Visvader:

- Australian Stem Cell Centre Symposium, Melbourne, September 2006 (Speaker)
Title: Breast stem cells and mammapoiesis
Author(s): Visvader JE
Abstract: none
- 3rd PacRim Breast and Prostate Cancer Meeting, Fraser Island, Queensland, Australia, October 2006 (Speaker)
Title: Stem cells in mammapoiesis and breast cancer
Author(s): Visvader JE
Abstract: none

François Vaillant:

- Familial Cancer – Research and Practice 2005, Annual Scientific Meeting, Couran Cove, Queensland, August 2006 (Speaker)
Title: Generation of a functional mammary gland from a single cell
Author(s): François Vaillant, Mark Shackleton, Kaylene J. Simpson, John Stingl, Gordon K. Smyth, Marie-Liesse Asselin-Labat, Li Wu, Geoffrey J. Lindeman, Jane E. Visvader
Abstract: refer appendix

Degrees supported in part by this award:

Dr Elgene Lim – Postgraduate Scholar (consumables component)

Development of animal models:

Development of xenograft model to assist growth of human breast tissue *in vivo*.

Funding applied for based in part on work supported by this award:

National Breast Cancer Foundation, Concept Award (A\$150,000)

CONCLUSION:

The work carried out to date has revealed that we will be able to prospectively identify subpopulations of epithelial cells using tissue derived from human breast (and tumor) tissue. Preliminary work, initiated with the support of the US DOD, has also shown that it will be possible to investigate these subpopulations using a range of markers, *in vitro* culture assays and xenograft models. Future studies will allow us to determine whether the subpopulations are indeed modified in the preneoplastic phase of cancer as well as determining whether breast tumors represent an expansion or modification of a specific subpopulation. This should form a basis for addressing the fundamental question of which cell type is the 'cell of origin' that gives rise to distinct forms of breast cancer, and could potentially provide a means to address the possibility that breast cancers contain a subset of 'tumor propagating cells' (cancer stem cells) (4-6). The elucidation of novel markers of cancer stem cells could potentially serve as novel therapeutic targets in breast cancer.

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- (4) Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. *Nature* 2001;414(6859):105-111.
- (5) Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci USA* 2003;100(7):3983-8.
- (6) Visvader JE, Lindeman GJ. Mammary stem cells and mammapoiesis. *Cancer Res* 2006;66(20):9798-801.

APPENDIX – meeting abstracts

Negative hormone receptor status of mouse mammary stem cells reminiscent of human basal breast tumours

Asselin-Labat M-L¹, Shackleton M¹, Stingl J², Vaillant F¹, Forrest NC¹, Eaves CJ², Visvader JE¹, Lindeman GJ^{1,3}

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The identification of at least five subtypes of breast cancer on the basis of gene profiling studies has generated considerable interest in identifying the cell types from which these phenotypically distinct tumour types arise. It is possible that normal progenitors, already restricted in their differentiative potential, become transformed, or that stem cells may be initially targeted with additional transforming events occurring in later progenitors. These possibilities prompted us to investigate the expression of ER α , PR, ErbB2/Her2, and EGFR/ErbB1 in normal mouse mammary stem cells.

Using the specific cell surface markers CD24 and $\alpha 6\beta 1$ -integrin, we have identified a distinct subpopulation of mammary cells that is enriched for mammary stem cells and expresses basal markers, and a subpopulation comprising luminal cells and stem cell-derived colony-forming cell progeny. The expression of key signalling molecules in these two subpopulations was examined. We found that the basal population, which is enriched in mouse mammary stem cells, did not express ER α , PR, or ErbB2/Her2 but did express epidermal growth factor receptor EGFR/ErbB1, whereas the subset of cells enriched for luminal cells expressed ER α (37% of cells) and PR (40% of cells) but not ErbB2/Her2 or EGFR/ErbB1. Ovariectomy confirmed the importance of estrogen signalling to luminal cell proliferation but had no effect on the size of the mammary stem cell-enriched population.

Thus, mouse mammary stem cells are ‘triple negative’ for ER α , PR, and ErbB2 and express basal markers. The phenotype of the mammary stem cell-enriched population recapitulates several characteristics of the poor-prognosis basal subtype of breast cancer, which is commonly observed in *BRCA1* tumours. One possibility is that the stem cell, or an early progenitor, is the ‘cell of origin’ that gives rise to the basal subtype of breast cancer.

Generation of a functional mammary gland from a single stem cell

François Vaillant^{1,2,*}, Mark Shackleton^{1,2,*}, Kaylene J. Simpson^{1,2}, John Stingl³, Gordon K. Smyth¹, Marie-Liesse Asselin-Labat^{1,2}, Li Wu¹, Geoffrey J. Lindeman^{1,2,*}, Jane E. Visvader^{1,2,*}

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The mammary gland can be functionally regenerated in mice by transplantation of epithelial fragments/cells, providing evidence for the existence of self-renewing, multipotential mammary stem cells. Using specific cell surface markers and flow cytometry, we have identified a distinct subpopulation that is enriched for mammary stem cells, demonstrated by transplantation into cleared fat pads at limiting dilution. Remarkably, a single mammary epithelial cell from this population, carrying the *lacZ* transgene, was found to generate a complete mammary gland *in vivo*. These cells contributed to both the luminal and myoepithelial lineages in transplanted virgin mammary glands, and extensive lobuloalveolar units were generated during pregnancy. Serial transplantation of the clonal outgrowths also yielded complete mammary glands. Together, these data establish that single cells from the enriched population have multipotential and self-renewing capacity, properties that are a hallmark of stem cells.

It will be of interest to determine whether the stem cell is a target of transformation in different mammary tumorigenesis models and whether analogous populations exist in human breast tissue and in breast cancer. Since BRCA1 *tumours* often exhibit a 'basal phenotype', which has been attributed to have stem cell properties, we intend studying whether the stem cell compartment is expanded or perturbed in preneoplastic breast tissue and tumours from BRCA1/2 carriers.