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Award Number: W81XWH-07-1-0558

TITLE: Cell fusion and Breast Cancer Metastasis

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REPORT DATE: September 2008

TYPE OF REPORT: Final

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

DISTRIBUTION STATEMENT: (Check one)

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
14-09-2008	Final	15 Aug 2007-14 Aug 2008

4. TITLE AND SUBTITLE
Cell Fusion and Breast Cancer Metastasis

5a. CONTRACT NUMBER
W81XWH-07-1-0558

5b. GRANT NUMBER
BC062124

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)
University of Iowa
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research
and Materiel Command
Fort Detrick, MD 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
This proposal had two overall objectives: 1) *Determine the frequency of breast cancer cell X host cell fusion events in experimental tumors*; and 2) *Measure the metastatic capacity of cells derived from fusion events*. We engineered murine mammary carcinoma cells with a reporter construct in which fluorescent protein (mCherry) expression was dependent upon Cre-mediated recombination. We demonstrated that delivery of Cre to reporter cells in vitro activated fluorescent protein expression demonstrating the utility of our system. We then injected cells orthotopically into the mammary fat pads of syngeneic hosts ubiquitously expressing Cre recombinase under control of the CMV promoter. We analyzed the resulting tumors for mCherry expression by FACS and histology. We did not detect any mCherry expressing cells by FACS nor did we see mCherry positive cells in tissue sections. We established that the FACS assay was sensitive enough to detect 1 in 1000 mCherry positive cells. Thus we were not able to identify cell fusion events in vivo using this approach.

15. SUBJECT TERMS
Cell fusion; metastasis

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	8	**19b. TELEPHONE NUMBER** *(include area code)*

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INTRODUCTION

Here we proposed to test the hypothesis that fusion between breast cancer cells and normal host cells occurs *in vivo* and contributes to metastasis. The rationale for this hypothesis is that prior studies have established that cancer cells can fuse with normal host cells and that this may enhance the malignant phenotype. These findings are of renewed interest in the context that stem cell-like cells may drive breast cancer progression (Al-Hajj et al., 2003). Yet, there are no studies to date that have addressed the possibility that cell fusion contributes to breast cancer progression or metastasis. To test our overall hypothesis we pursued the following specific aims: 1) Determine the frequency of breast cancer cell X host cell fusion events in experimental tumors. 2) Measure the metastatic capacity of cells derived from fusion events.

BODY

Regulatory Review of Animal Studies

Completed institutional review, ACURF#052026.

1) *Determine the frequency of breast cancer cell X host cell fusion events in experimental tumors.*

Task 1.1 Complete characterization of breast cancer reporter cell line

To test the fusion reporter construct we designed called LSL-GFP (Fig. 1A), we stably transfected B16 melanoma cells with this vector and exposed them to Cre-adenovirus. This activated GFP expression as detected by FACS (Fig. 1B) and allowed growth in the presence of ganciclovir (Fig. 1C). Subsequent to these experiments we obtained a different fluorescent protein, mCherry, with superior spectral characteristics for in vivo imaging. We replaced green fluorescent protein in the reporter construct with mCherry and performed similar experiments with 4T1 mammary carcinoma cells (see Fig. 4 for example).

Task 1.2 Generate xenograft tumors in CMV-Cre mice

We generated tumors in mammary fat pads in CMV-Cre mice with 4T1-LSL-mCherry cells (Fig. 2). Tumors were allowed to grow to endpoint size (~3 weeks) at which time mice were euthanized and tumors isolated for analysis.

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Task 1.3 Isolate and characterize GFP-positive and GFP-negative cell line

We isolated cells from 4T1 tumors and subjected them to FACS analysis. 10^6 live cells were analyzed from each tumor sample. No mCherry-positive cells were identified in this analysis (Fig. 3). We wished to know the sensitivity limits of the FACS protocol that we used, so we titrated mCherry-positive cells into mCherry-negative cells. We determined that in an analysis of 106 cells, our detection limit was ~1:1000 (Fig. 4).

Task 1.4 Evaluate tumor sections for GFP-positive cells

No mCherry-positive cells were identified in tumor sections.

2) *Measure the metastatic capacity of cells derived from fusion events.*

Task 2.1 Evaluate metastatic potential of GFP-positive and GFP-negative cell lines.

Because we did not isolate any mCherry-positive cells from task 1.3 above, we could not perform task 2.1.

KEY RESEARCH ACCOMPLISHMENTS

- Generated reporter cell line with optimal fluorescent protein (mCherry) expression.
- Demonstrated that mCherry expression was dependent on Cre in vitro.
- Generated orthotopic tumors in CMV Cre mice with reporter cell line.
- Isolated cells from tumors and attempted to identify mCherry positive cells by FACS, none detected.
- Prepared tissue sections from tumors, examined by fluorescence microscopy-no mCherry positive cells detected.
- Established sensitivity limits of FACS assay at 1:1000.

REPORTABLE OUTCOMES

- Abstract presented at 2008 ERA of HOPE meeting, Baltimore MD
- Reporter cell line developed

CONCLUSION

We were not able to obtain any support for our hypothesis in these studies. This is because either cell fusion does not occur in the breast cancer model that we examined or technical limitations precluded us from detecting these events. To address the latter, we established sensitivity limits in our assay of 1 mCherry positive cells in 1000 mCherry negative cells. It is possible that cell fusion events are less frequent that 1:1000 in which case our approach is not sensitive enough to detect them. We could improve sensitivity through rare event analysis protocols or increasing the number of cells evaluated by FACS.

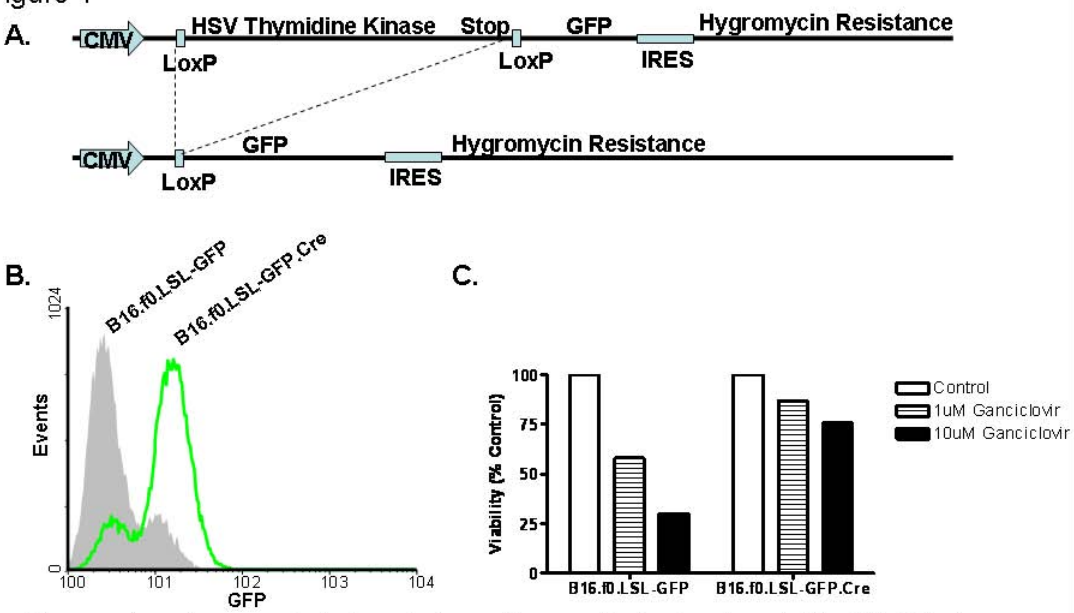
REFERENCES

Al-Hajj, M., Wicha, M. S., Benito-Hernandez, A., Morrison, S. J., and Clarke, M. F. (2003). Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A *100*, 3983-3988.

APPENDICES
None.

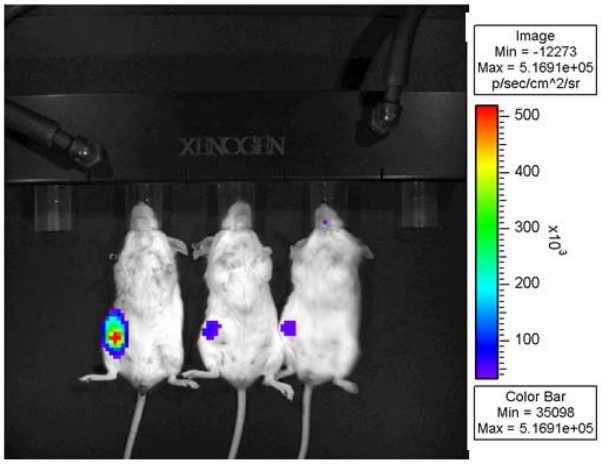
SUPPORTING DATA

Figure 1



Fluorescent reporter expression is dependent upon Cre recombination in cells containing LSL-GFP. A. Structure of LSL-GFP vector before (top) and after (bottom) Cre-mediated recombination between LoxP sites. B. FACS analysis of B16.f0.LSL-GFP and B16.f0.LSL-GFP.Cre showing that GFP expression is dependent upon Cre activity. C. Cells from B were treated with either vehicle control, 1, or 10μM ganciclovir and viability assays were performed showing that after Cre recombination, thymidine kinase is efficiently removed and cells are resistant to ganciclovir. The Y axis represents viability in terms of percent of control treated cells. CMV = cytomegalovirus promoter; HSV = Herpes Simplex Virus; GFP = green fluorescent protein; IRES = Internal Ribosomal Entry Site.

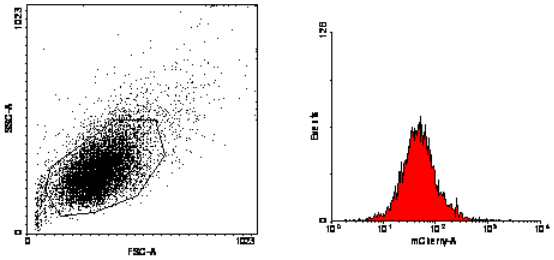
Figure 2



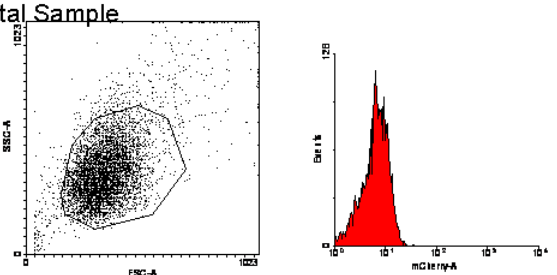
Female CMV-Cre mice were injected (in the 4th mammary fat pad) with 5,000 live 4T07 cells containing the Cre-dependent mCherry reporter and constitutively expressing firefly luciferase. Tumor growth was monitored using bioluminescence imaging (mice receive 15mg/kg does of D-luciferin intraperitoneal and imaged in IVIS-100, Xenogen). This image is representative of 3 mice injected with positive control mCherry-positive cells @ two weeks post injection.

Figure 3

Positive Control Sample

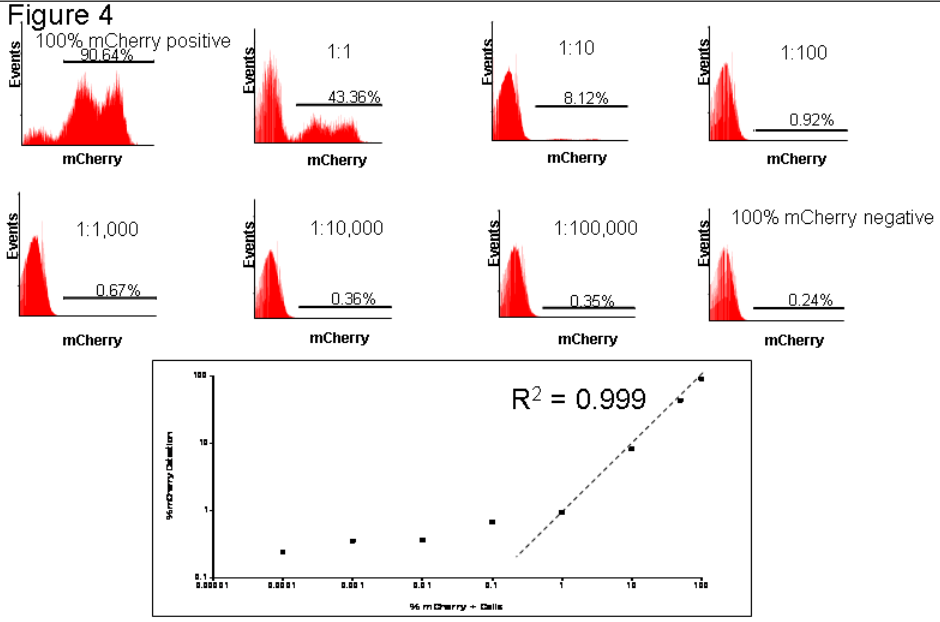


Experimental Sample



Primary tumors were removed from all mice after they had reached size limitations. Tumor tissue was treated with collagenase to produce a single cell suspension. After 70um filtration of suspensions, FACS was performed to detect and sort mCherry expressing cells. In the histograms above, tumor tissue formed from injection of mCherry-positive cells appear uniformly positive for mCherry expression, whereas no mCherry expression was detected in any of the experimental tumors (Cre-dependent mCherry tumors). For each sample, 1 x 10^6 live cells were analyzed.

Figure 4



4T07 mouse mammary cancer cells were stably transfected with Cre-dependent mCherry reporter construct. mCherry positive cells were obtained via FACS following Cre-Adenoviral infection of the parental population ("100% mCherry negative). mCherry-positive and –negative cells were mixed at the ratios listed above each histogram and then analyzed via flow cytometry to determine sensitivity of detection. For each sample, 1 x 10^6 live cells were analyzed; a value amenable to live cell counts of fresh tumor tissue suspensions.