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PRINCIPAL INVESTIGATOR: Chung-Cheng Hsieh

CONTRACTING ORGANIZATION: University of Massachusetts Medical Center
Worcester, MA 01655

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14. ABSTRACT Breast density can be assessed by whole mount carmine alum staining of the fourth inguinal mammary glands according to the method reported by Brisken et al (2002) and counting the number of terminal end buds. Mammary glands of pregnant C57Bl/6J mice were found to have significantly more terminal end buds than virgin mice. Flow cytometric analyses of dissociated cells from mammary glands clearly identified a sub-population of cells that are CD49f ⁺ and CD24 ⁺ , putative markers of mammary stem/progenitor cells.					
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Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	5
Reportable Outcomes.....	5
Conclusion.....	6

INTRODUCTION

The objective of this proposed study is to understand the biological determinants of mammographic density, a strong predictor of human breast cancer risk. Our hypothesis is that the *in utero* levels of mitogens, such as IGF-1, drive an increased number of breast stem/progenitor cells in the mammary gland, which in turn results in a more extensive epithelial mammary tree formation, i.e., an increase in mammographic density. Since breast stem/progenitor cells are the cell population within the mammary gland that is presumed to be susceptible to malignant transformations, dense breast with elevated levels of stem/progenitor cells, should have a higher risk of becoming malignant. The study hopes to correlate *in utero* levels of IGF-1 administered to the mother with the birth weight, breast/mammographic density and number of breast stem/progenitor cells of the offspring.

BODY

Timetable of research accomplishments as outlined in the Statement of Work:

- a. Obtain approval for animal research; develop and validate microparticle delivery vehicle generation. (Months 0-3)
- b. Obtain IGF-1^{m/m} and control mice and begin breeding. (Months 3-6)
- c. Perform preliminary studies to quantitate and validate breast stem/progenitor FACS assay and breast density assay. (Months 3-6)
- d. Perform experiments on effect of *in utero* IGF-1 treatment on breast stem/progenitor pools and breast density using cohorts of bred mice. (Months 8-32)
- e. Compile data, perform statistical analysis and write manuscript. (Months 32-36)

Progress report:

Since the launching of the project, we have obtained approvals for animal research from the Institutional Animal Care and Use Committee (IACUC), University of Massachusetts Medical School (UMMS) in May 2008 and from the Animal Care and Use Review Office (ACURO), Department of The Army in June 2008 (Task a).

We performed preliminary studies to quantitate and validate breast density using histological assays, and by *in vivo* and explant imaging (Task c). Histologically, we stained mammary glands from virgin and pregnant C57Bl/6J mice using whole-mount staining protocols as reported by Nandi (1958), Fata et al (1999) and Brisken et al (2002). Although all three methods gave good staining of mouse mammary glands as visualized under a microscope, with a greater number of terminal end buds from mammary glands of pregnant mice (**mean \pm standard error of the mean (SEM) of terminal end bud-like structure counts of 626 ± 25.4 , n = 4**) than from virgins (**5.5 ± 0.5 , n = 2**), the method as reported by Brisken gave the best staining of terminal end buds (**Fig 1**). We improved on the method by cover-slipping the slides using Permount (Fisher Scientific) whereby the stained mammary glands are preserved indefinitely and can be viewed microscopically for analysis at any time. We also explored whether breast/mammographic density can be measured radiographically using a Nanospect/CT *in vivo* animal imager, available at the Small Animal Imaging Core Facility, UMMS. To do this, we wrote a separate animal protocol and obtained approval from IACUC, UMMS in May 2009. Although the device could image the mammary glands both *in vivo* and as explants, it could not detect the difference in radiographic density of mammary glands between virgin and pregnant mice.

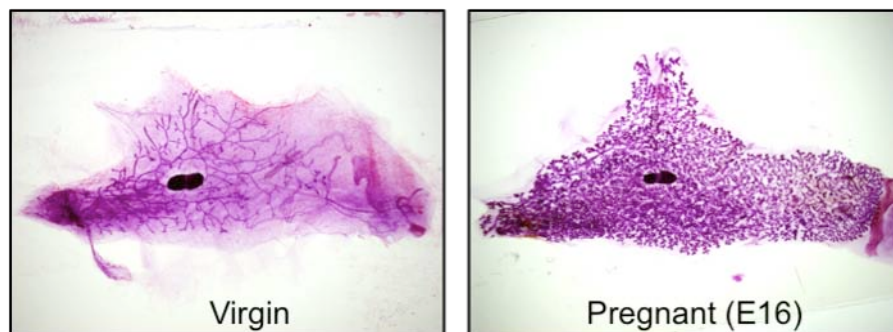


Fig 1. Whole mount fourth inguinal mammary glands of C57Bl/6J mice stained with carmine alum solution, mounted and cover-slipped on glass slides using Permount showed that a mammary gland from a virgin mouse (left panel) is “less dense” than that of a E16 pregnant gland (right panel); both glands are from 8 week-old mice.

We performed preliminary studies to quantitate and validate stem/progenitor using flow cytometric assay (Task c). Using protocols adapted from StemCell Technologies Inc. (Vancouver, Canada) and Stingl et al (2006), we successfully detected a sub-population of cells from mammary glands that are CD49f⁺ and CD24⁺, putative markers of mammary stem/progenitor cells. **In mammary glands of wild type C57Bl/6J control mice, the CD49f⁺/CD24⁺ population ranged from 0.42 to 1.5% of total cells, with a mean \pm SEM value of 1.06 \pm 0.23% (n = 4) (Fig 2).** We are presently investigating whether we can also detect other putative markers of mammary stem/progenitor cells, such as CD24⁺/CD29⁺ (Shackleton et al, 2006) and CD24⁺/CD29⁺/CD49f⁺ (Visvader and Lindeman, 2006).

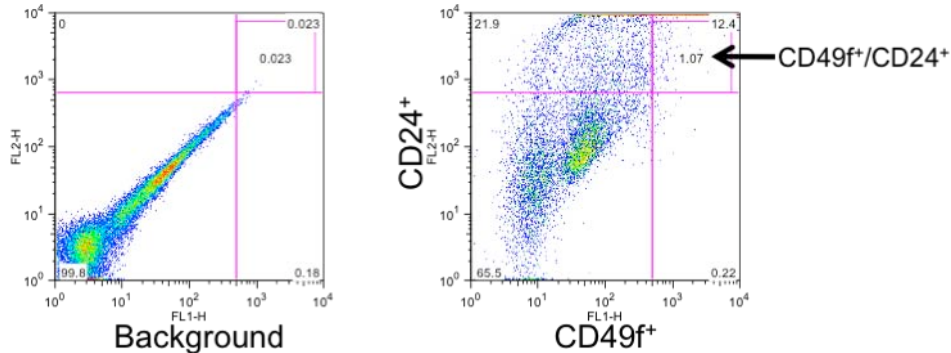


Fig 2. Flow cytometric dot plots showing the identification of a population of CD49f⁺/CD24⁺ cells in the upper right quadrant (arrow) from C57Bl/6J dissociated mammary cells.

Homozygous *Igf1^{m/m}* mice are being prepared for the project (Task b). While this is being carried out, investigations are being carried out using wild type mice (Task b and d). We are performing studies using wild type C57Bl/6J mice to determine the optimal dose of IGF-1 that can confer an *in utero* effect on the birth weights and mammary stem/progenitor cells of the offspring. We found that the original proposal of delivering IGF-1 microparticles trans-placentally into pregnant mice to be cumbersome and caused unnecessary trauma to the mice. We now propose to administer IGF-1 into pregnant mice via daily intra-peritoneal (ip) injections on embryonic days (E) 10 to 16, inclusive. We have written a minor amendment for this proposed change that was approved by IACUC, UMMS in June 2009. We have investigated 3 concentrations of IGF-1 (5 μ g/kg, 10 μ g/kg and 15 μ g/kg of maternal body weight) with vehicle for controls and the results are being analyzed.

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KEY RESEARCH ACCOMPLISHMENTS

- Breast/mammographic density was successfully assessed by whole mount carmine alum staining of the fourth inguinal mammary glands.
- Coverslipping using Permount of whole mount carmine alum stained mammary glands preserved the histological staining indefinitely.
- A putative sub-population of mammary stem/progenitor cells that were CD49f⁺ and CD24⁺ was successfully identified from dissociated cells of mammary glands.

REPORTABLE OUTCOMES

- By assessing breast density by whole mount carmine alum staining of the fourth inguinal mammary glands, mammary glands of pregnant C57Bl/6J mice were found to have significantly more terminal end buds than virgin mice.

- Flow cytometric analyses of dissociated cells from mouse mammary glands clearly identified a sub-population of cells that are CD49⁺ and CD24⁺, putative markers of mammary stem/progenitor cells.

CONCLUSIONS

This project is progressing according to plan.