AD_____

Award Number: W81XWH-04-1-0666

TITLE: Mammary Stem Cell Susceptibility to Carcinogenesis

PRINCIPAL INVESTIGATOR: Sue C. Heffelfinger, M.D., Ph.D.

CONTRACTING ORGANIZATION: University of Cincinnati
Cincinnati, OH 45267-0553

REPORT DATE: July 2005

TYPE OF REPORT: Final

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-07-2005	Final	1 Jul 2004 - 30 Jun 2005

4. TITLE AND SUBTITLE
Mammary Stem Cell Susceptibility to Carcinogenesis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04-1-0666

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Sue C. Heffelfinger, M.D., Ph.D.

E-mail: sue.heffelfinger@uc.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Cincinnati
Cincinnati, OH 45267-0553

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 color plates: All DTIC reproductions will be in black and white.

14. ABSTRACT
Abstract follows.

15. SUBJECT TERMS
Rat, mammary gland, stem cell, mammosphere

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	19	

13. Abstract (Maximum 200 Words) *(abstract should contain no proprietary or confidential information)* Mammary tumor formation is thought to be the consequence of transformation of undifferentiated pluripotent stem cells. Little is known about the character of mammary gland stem cells. Examination of mammary gland development has indicated that there may be three distinct types of stem/progenitor cells (S/PC); those which only form ductal structures, those which only form alveolar structures and those which form all mammary gland structures. The goal of this work was to isolate S/PC from rat mammary glands using the recently described techniques of mammosphere formation and Hoechst dye-exclusion. Additionally, we sought to characterize the S/PC populations as to their ability to differentiate in solid matrix and their expression of certain antigens. Lastly, we aimed to determine whether the susceptibility of mammospheres (containing S/PC and transit amplifying cells TAC) to carcinogens varied compared to the intact mammary epithelial organoid (primarily TAC). We cultured rat mammospheres and demonstrated two hallmarks of S/PC, their ability to self renew and to differentiate in solid matrix. We also isolated side-population (SP) cells which excluded Hoechst dye and reformed mammospheres. Immunostaining results were similar for organoids and mammospheres. Further work is needed to clearly assess which cell type is necessary for carcinogenesis.

Table of Contents

INTRODUCTION

A major unanswered question in breast cancer research is the cell of origin. The mammary gland is comprised of adult stem cells from which all other structures are derived, progenitor cells (PC) that can differentiate into either ductal or alveolar structures, transit amplifying cells (TAC) which are differentiated but are still capable of division, and fully differentiated cells which can no longer divide [1]. Two schools of thought currently exist seeking to explain the cell of origin for cancer. One hypothesis is that all cancers are derived from stem/progenitor (S/PC) cells, which confers upon them an immortal or extended lifespan and the ability to undergo partial differentiation along one or more phenotypic pathways. Tumor cells though are often quite differentiated which leads to the second hypothesis: TAC may parent tumors and dedifferentiate in ways which lead to expanded lifespan (immortality). TAC are similar to tumors in that both are normally rapidly proliferating cells. We hypothesize that S/PC parent tumors; however initiated TAC are necessary modifiers of carcinogenesis through the development of a permissive microenvironment for tumor formation.

We sought to study this in an in vitro setting using S/PC cells enriched through rat mammosphere culture techniques and TAC from rat mammary epithelial organoid culture techniques. Since many stem/progenitor enrichment protocols utilize immunosorting based on cell-surface proteins, we sought to characterize both S/PC and TAC from rat mammary epithelial cells using antibodies shown by others to be expressed by mammary S/PC. Another characteristic shared by stem cells is their ability to exclude dyes like Hoechst or rhodamine due to increased expression of membrane transporter proteins [2]. We sought to characterize the cells present in mammospheres as to their ability to exclude Hoechst dye termed the side-population (SP). While mammary S/PC have been characterized from human and mice tissue, these data are the first reports from rat mammary glands.

BODY

Statement of Work Product: Repeat studies published by other investigators (mice and human tissue) using rat mammary glands to culture mammospheres and show these structures are enriched for stem/progenitor cells (S/PC).

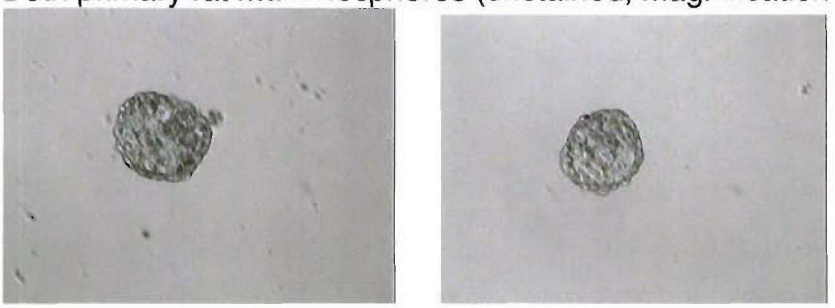
Mammosphere culture

Stem/progenitor cells (S/PC) are defined by their ability to self-renew and regenerate. In order to propagate sufficient rat mammary stem/progenitor cells to study and characterize, we utilized the methodology employed by others [2] using human mammary epithelial cells. Mammary tissue from virgin rats was dissociated mechanically and enzymatically to rat mammary epithelial (RME) organoids, as previously described [3,4]. Organoids were then digested enzymatically to single cells using 0.05% trypsin (Cellgro, Mediatech Herndon, VA). Single cells were sieved sequentially through 70 μm and 40 μm cell strainers (BD-Falcon) to obtain a single cell suspension. An aliquot of this suspension was used to confirm that the number of single cells was >98% in all experiments. The cell suspension was resieved when the percentage of single cells was lower than 98%. Single RME cells were plated in serum-free mammary epithelial growth medium (MEGM excluding bovine pituitary extract, Cambrex, Walkersville, MD) and supplemented with B27 (Invitrogen Carlbad, CA), and 20 ng/mL bFGF (RD, Minneapolis, MN). Cells in MEGM were plated onto agarose-coated Petri dishes at a density of 20,000 viable cells/mL in primary culture and 1000

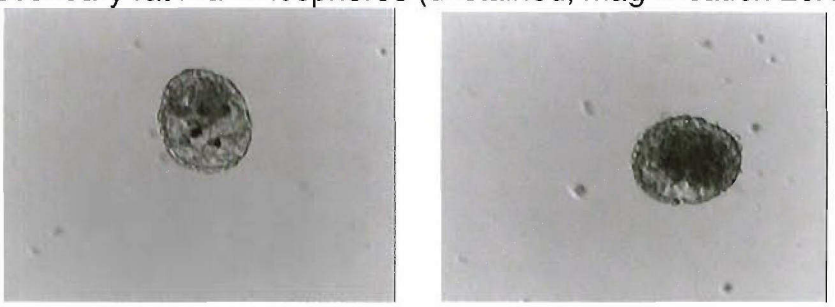
cells/mL in passages. Mammospheres were collected by gentle centrifugation after 7–10 d and dissociated enzymatically (0.05% trypsin). Dissociated cells were sieved sequentially through 70 μm 40 μm cell strainers. An aliquot of this suspension was used to confirm that the number of single cells was >98% in all experiments. The cell suspension was resieved when the percentage of single cells was lower than 98%.

As others have shown in neurons and mammary cells from humans and mice, we were able to culture mammospheres from single rat mammary epithelial cells in a suspension culture in low-attachment (agarose-coated) dishes.

Both primary rat mammospheres (unstained, magnification 20X)



and secondary rat mammospheres (unstained, magnification 20X)



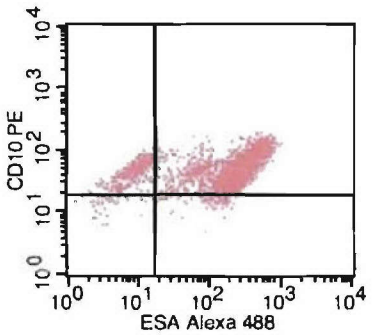
are similar to others reported [2]. We were successful in culturing and passing these to produce secondary and tertiary mammospheres demonstrating the self-renewal capability. These results are similar to previous reports of mammosphere formation from human mammary cells [2]. The rat mammospheres were estimated to contain between 150 and 300 cells. Typically, 2-4 spheres were obtained per 1000 plated cells following mechanical and enzymatic dispersement and passing cells through sequential sieves. This was true for all passages and is similar to previous reports [2]. It is estimated that between 25-50% of dissociated cells from rat mammary epithelial organoids or mammospheres recovered and regenerated new mammospheres.

Characterization of cells from mammospheres and organoids using antibody staining and FACS analysis

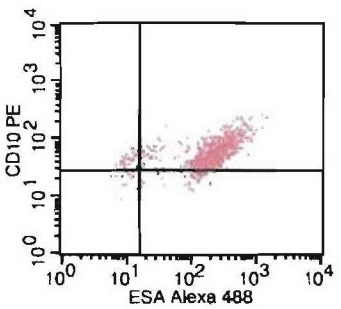
Since others [2] have published immunostaining of enriched human mammary S/PC, we sought to characterize the cell-surface proteins which might be used to distinguish between rat mammary S/PC and TAC using combinations of common antibodies to epithelial cells. Single cells from dissociated mammospheres or from dissociated mammary organoids were incubated with one or more antibodies then

stained with 1µg/mL 7-AAD (Sigma) for viability, before analysis using FACS Calibur (Becton Dickinson). The following antibodies were tested: Sca-1 anti-mouse rat IgG_{2a} (RD Clone 177228) biotinylated, Sca-1 anti-mouse rat IgG_{2a} (BD clone E13-161.7) PE conjugated, CD24 anti-human, mouse IgG2a (abcam clone 219) unconjugated, CD44 anti-rat, mouse IgG_{2a} (BD clone OX-49) unconjugated, ESA anti-human mouse IgG_1 (US Biological clone E3414-08) unconjugated, CD10 anti-human mouse IgG_1 (NeoMarkers Clone 56C6) unconjugated. The unconjugated antibodies were conjugated to fluorophores using Zenon Labeling Kits (Molecular Probes, Eugene, OR). ESA was conjugated with Alexa-488, CD44 was conjugated with APC, CD24 was conjugated with APC and CD-10 was conjugated with PE. The biotinylated Sca-1 Ab was labeled with Streptavidin-APC conjugate using a kit (Molecular Probes). Reactivity of these antibodies to rat tissues was confirmed first using manufacturer's recommendations.

When cells from dissociated organoids (below left) or dissociated mammospheres (below right) were incubated with both ESA-Alexa 488 and CD10-PE antibodies, approximately 85% of the live cells were positive for both antibodies. The similarity of both dot plots indicated that while S/PC are present in these mammospheres, a majority of cells are TAC, the primary components of organoids. The expected enrichment of S/PC in the mammospheres was not evident using immunostaining with these antibodies.

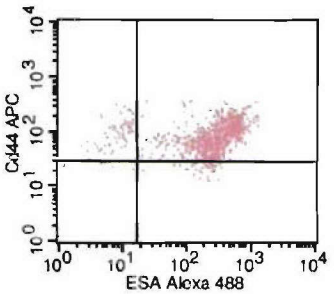


Quad	% Gated
UL	9.87
UR	85.96
LL	1.16
LR	3.01

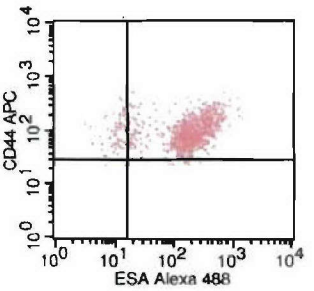


Quad	% Gated
UL	4.40
UR	84.67
LL	2.03
LR	8.91

Similarly, when cells from dissociated organoids (below left) or dissociated mammospheres (below right) were incubated with both ESA-Alexa 488 and CD44-APC, approx. 88-90% of the live cells were positive for both antibodies. The similarity of these dot plots indicated that while S/PC are present in these mammospheres, immunostaining with these 2 antibodies was not able to distinguish differences in cellular populations within mammary organoids and cultured mammospheres.

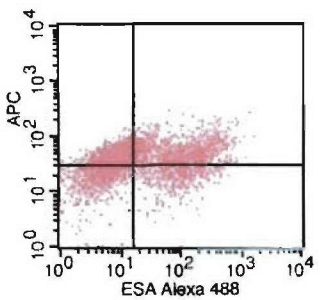


Quad	% Gated
UL	6.60
UR	88.04
LL	0.52
LR	4.85



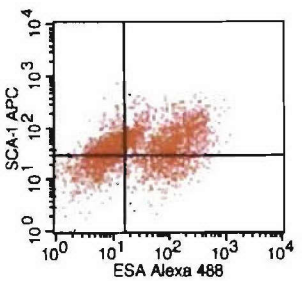
Quad	% Gated
UL	7.18
UR	91.05
LL	0.39
LR	1.38

Dontu et al [5] have published that when tumor cells are immunosorted using ESA/CD44/CD24, the tumor SC are ESA+/CD44+/CD24-. We sought to evaluate this combination of antibodies in the mammospheres. When cells from dissociated mammospheres (below) were incubated with both ESA-Alexa 488 and CD24-APC, approximately 31% of the live cells were positive for both antibodies and 12% of the cells were positive for ESA and negative for CD24.



Quad	% Gated
UL	38.03
UR	31.43
LL	18.56
LR	11.98

Others [6] have shown that mammary S/PC immunostained positively with SCA-1 (stem cell antigen-1) and also regenerate the mammary gland in vivo. When cells from dissociated rat mammospheres (below) were incubated with both ESA-Alexa 488 and Sca-1-APC approximately 36% of the live cells were positive for both antibodies. Though not shown below, when organoids were dispersed and labeled with these same antibodies, the percentage of cells staining positive for both antibodies was similar (36-40%). These data indicate that both mammospheres and organoids contain similar populations of cells which appear to be primarily TAC.



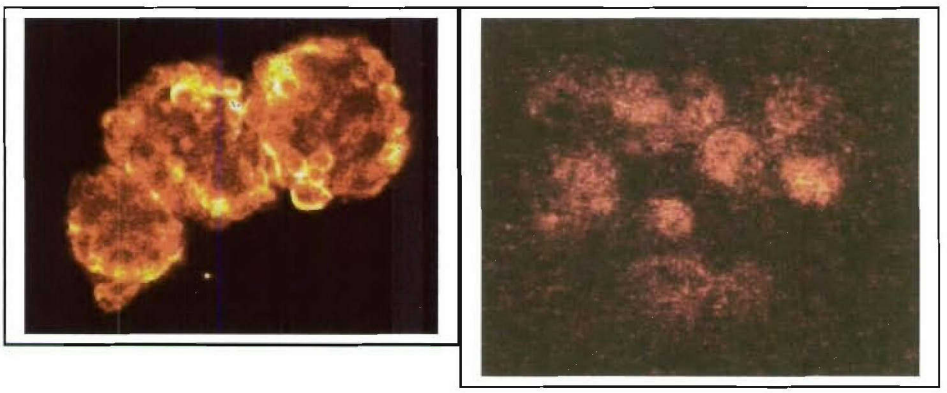
Quad	% Gated
UL	35.02
UR	36.38
LL	17.61
LR	10.99

The data (dot plots) shown above from the FACS Calibur indicate that these rat mammospheres are enriched for S/PC but they are also composed of TAC and are nearly indistinguishable from cells of organoids when treated with the same combinations of antibodies used by others to characterize S/PC [2,5,6].

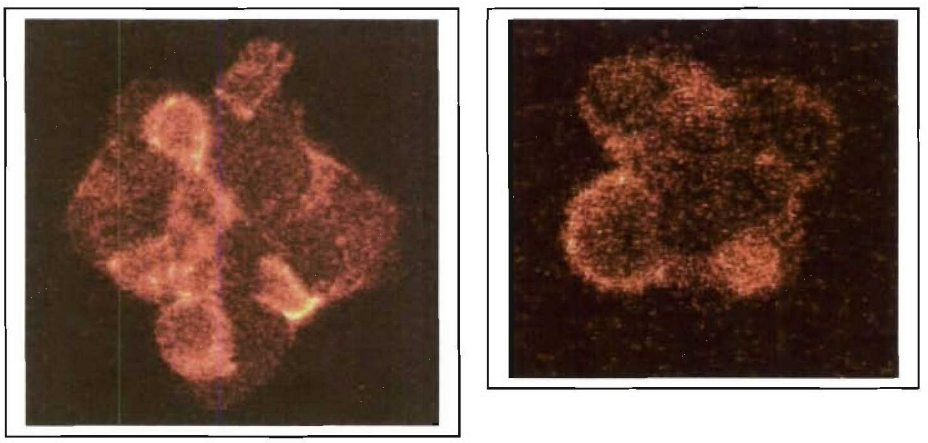
Antibody staining of intact mammospheres

Based on previous work with human S/PC from mammospheres, S/PC typically stain positive for certain antibodies such as CD10, cytokeratin 6 and alpha-6-integrin [2]. We sought to determine whether this was true for rat mammospheres. In order to visualize which specific cells within the mammosphere were staining with these antibodies, mammospheres were collected by gentle centrifugation (800 rpm), washed with PBS, dried onto slides, fixed, stained with antibodies and photographed (100X).

Cells within primary spheres stained positive for keratin 5/6/18 (below left), CD44 (below right)



CD10 (below left) and ESA (below right).

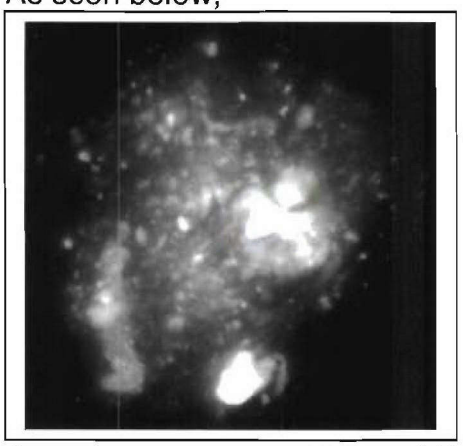


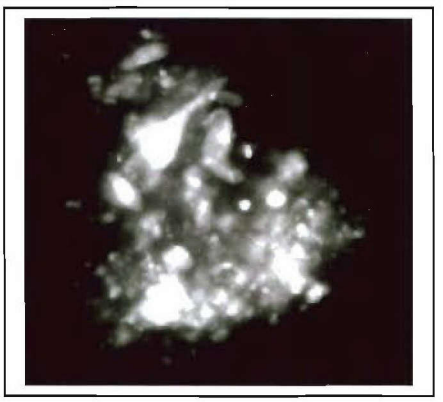
These staining experiments seemed to indicate more cells staining positive for these antibodies than would be expected if the antibodies were staining primarily the S/PC. Additionally, other reports indicated a localization of positively staining S/PC cells within the center of human mammospheres [2] not seen in the rat mammospheres.

Hoechst staining of intact mammospheres

In order to confirm that cultured mammospheres were composed of side population (SP) cells, we first incubated intake spheres with Hoechst 33342. Primary mammospheres were collected by gentle centrifugation, resuspended in fresh MEGM containing 2.5 µg/mL Hoechst 33342 and incubated for 45 min at 37°C. Following the incubation, mammospheres were collected by gentle centrifugation washed with cold PBS then placed onto slides for immediate microscopy (20X).

As seen below,



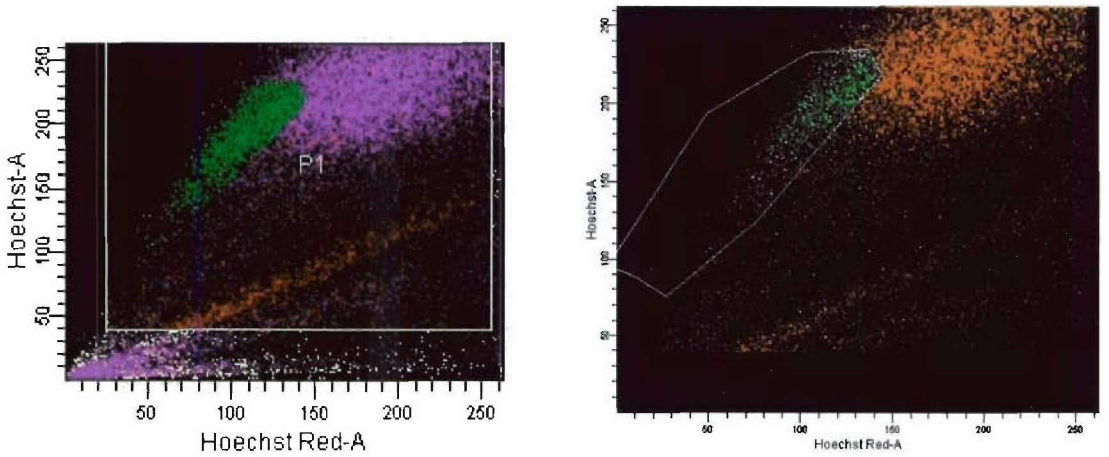


the spheres were a mixture of Hoechst-excluding cells (side population SP cells) and cells which did not exclude Hoechst (non-SP cells). This provided data to pursue the next step of isolating these SP (Hoechst-excluding cells) using FACS analysis and to test their viability and capability for regeneration.

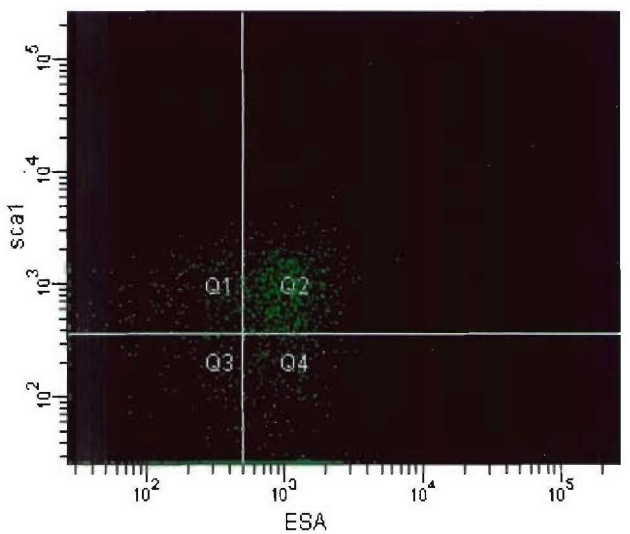
Hoechst 33342 staining and Flow Activated Cell Sorting (FACS) analysis for Side Population (SP) Cells

Since the mammospheres appear to be composed primarily of TAC which could not be easily separated using the antibodies we have tried, we sought to separate SC from the TAC using Hoechst-exclusion. This technique has been successfully used for separation of SC within bone marrow [6]. The Hoechst staining was performed as previously described [2,6,7] FACSVantage SE (Becton Dickinson). Briefly, cells from dissociated mammospheres or from dissociated rat mammary epithelial organoids were sequentially sieved through 70 μm and 40 μm cell strainers, resuspended in medium (100,000 cells/mL) and allowed to recover at 37°C for 1 h before treatment with Hoechst. Hoechst 33342 (Sigma) was added at a final concentration of 2.5-4.5 μg/mL and incubated at 37°C for 45 min. Following incubation, cells were placed on ice, washed with ice-cold PBS, and resuspended in ice-cold PBS. When staining with antibodies was performed, cells were incubated with Ab, on ice, then washed with ice-cold PBS, resuspended in PBS on ice. Before cells were taken for FACS, they were stained with 1μg/mL propidium iodide (Sigma) for viability, then analyzed and sorted by FACS using FACS VANTAGE SE (Becton Dickinson). The Hoechst dye was excited at 350 nm and its fluorescence was measured at two wavelengths, 450/20 BP filter Blue and 675 EFLP optical filter Red as described previously [7]. Sorted cells were resuspended in mammosphere medium and plated onto agarose-coated dishes or resuspended in serum-free medium-5% FBS.

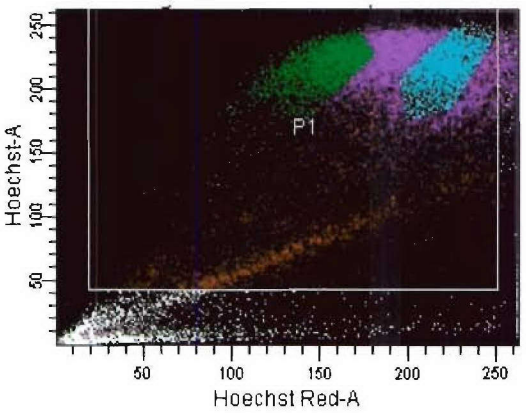
FACS analysis of Hoechst-33342 exclusion (dot plots) reveals a population of cells (SP) which effluxes Hoechst dye (termed the arm) as well as a population of non-excluding cells (non-SP) cells termed the head.

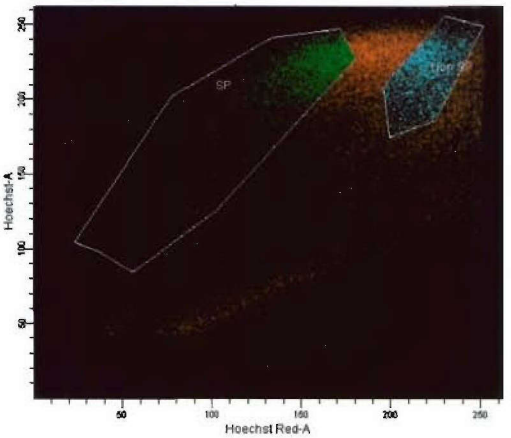


The SP cells were gated further, approximately 32% stained positively for both ESA-Alexa 488 and Sca-1-APC antibodies (seen below). This dot plot was similar to the dot plot obtained for the entire mammosphere population (shown above).



Subsequent analyses indicated that the side-population (SP-green highlight) fraction of cells represented ~10-16% of the gated live cells while the non-side population (non-SP light blue highlight) fraction represented approximately 80% of the gated live population.



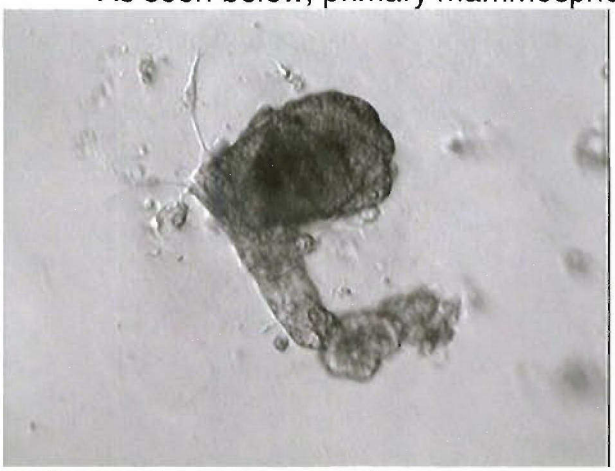


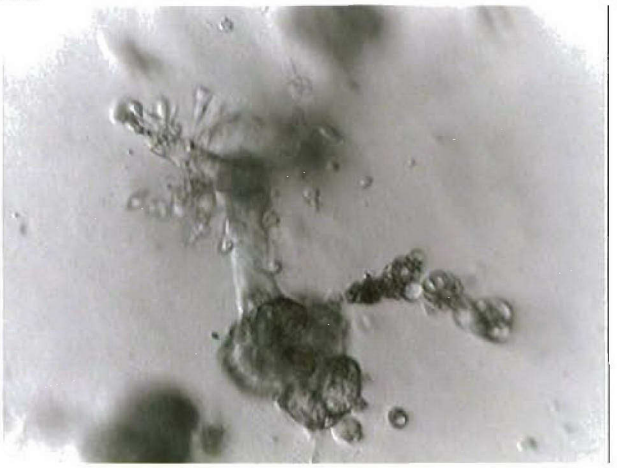
This analysis is similar to other reports of mouse mammary epithelium [6] and mouse marrow [7]. Sorted SP and non-SP cells were resuspended in mammosphere medium and plated onto agarose-coated dishes or resuspended in medium containing hormones and 5% FBS onto matrigel as single cells in 96 well plates. The latter failed to successfully grow under these conditions despite repeated attempts unlike other reports [2]. The cells plated in suspension did, however, grow and regenerated mammospheres. From ~60,000 cells collected as SP (Hoechst-excluding), approximately 6-8 spheres formed and of ~90,000 non-SP (non-Hoechst excluding) cells plated, one mammosphere formed. These results demonstrated functionally that SP cells are S/PC cells capable of regeneration and renewal. Unfortunately, the presence of sphere-forming cells in the non-SP fraction demonstrates that the conditions used in the Hoechst-exclusion may not be optimal for the rat mammospheres.

Differentiating culture conditions

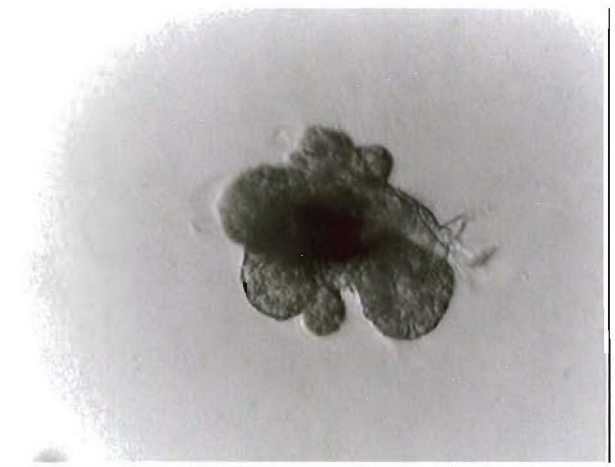
Since adult S/PC are characterized by their ability to generate the entire repertoire of cell types found in a tissue [8], we sought to characterize rat non-adherant mammospheres for their enrichment of S/PC. In order to evaluate the capability of rat mammospheres to differentiate into alveolaer and/or ductal structures, primary and secondary mammospheres were plated on both collagen and Matrigel in medium enriched with both hormones and 5% FBS.

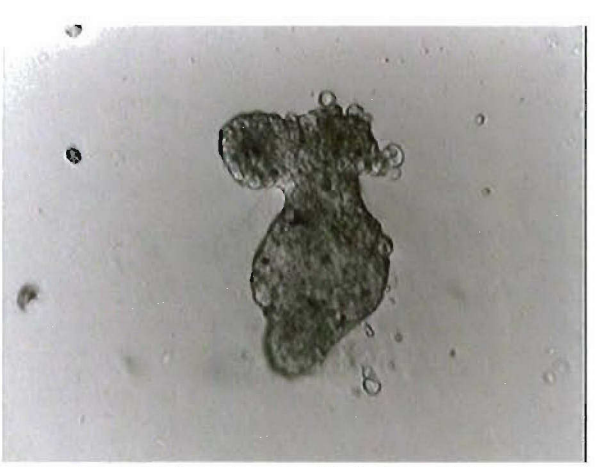
As seen below, primary mammospheres





and secondary mammospheres





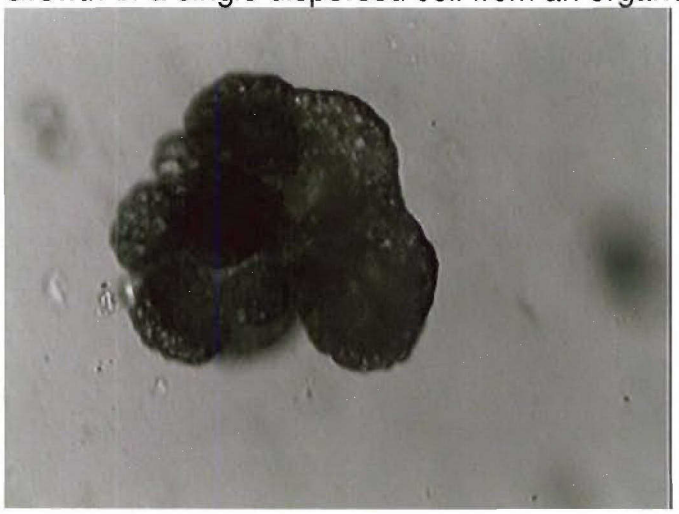
are capable of differentiating to both alveolar and ductal structures. This provides confirming evidence that these mammospheres contain stem cells (S/PC) capable of differentiation on solid substrate, a hallmark of stem cells.

Statement of Work Product: Through in vitro studies, show which cell types (S/PC or TAC) are required for microtumor formation following initiation with a polyaromatic hydrocarbon.

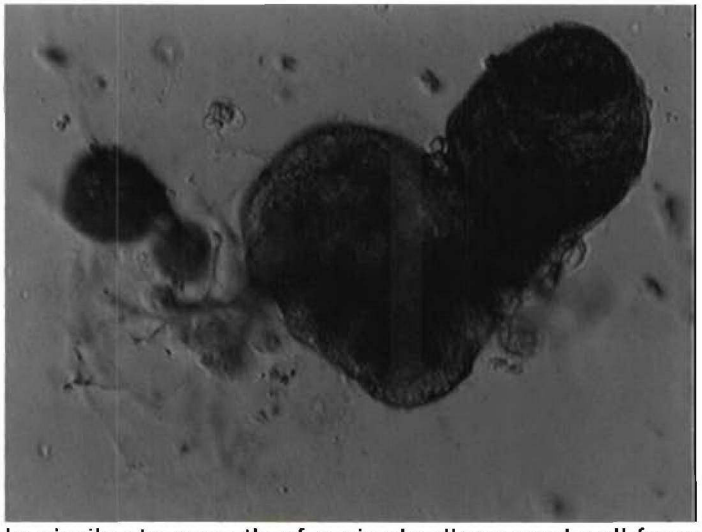
Initiation of mammospheres in suspension and organoids in collagen with chemical carcinogens to develop "microtumors"

Even though our data collected to date indicated that rat mammospheres did contain S/PC, they were composed of TAC, similar to starting organoids, we sought to evaluate whether initiation of mammospheres with a chemical carcinogen would differ from initiation of mammary organoids. Both were treated for 24 hours with either 100 nM benzo[a]pyrene (BaP) or 100 nM dimethylbenz[a]anthracene (DMBA) or dimethylsulfoxide (DMSO) control. The cultures were then enzymatically digested to single cells and plated on collagen or Matrigel in medium supplemented with 5% FBS. Cultures were maintained for 2-3 weeks where growth and differentiation were evident though not necessarily as a result of treatment with carcinogen. The results were similar with mammospheres or organoids as seen below.

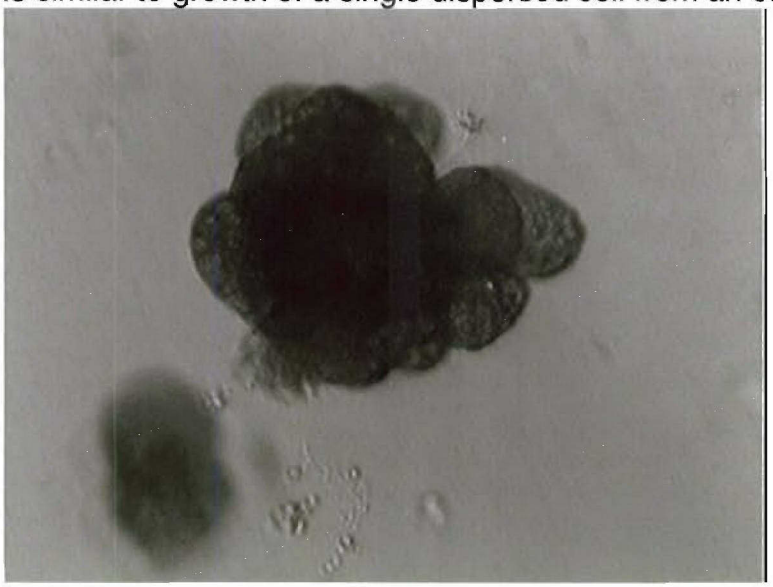
Growth of a single dispersed cell from an organoid treated with DMSO (20X)



Is similar to growth of a single dispersed cell from an organoid treated with BaP (20X)

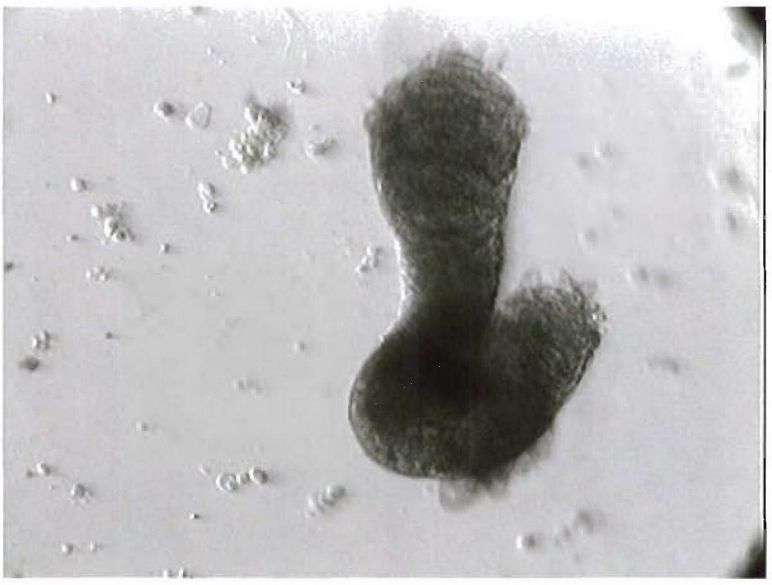


Is similar to growth of a single dispersed cell from an organoid treated with DMBA (20X)

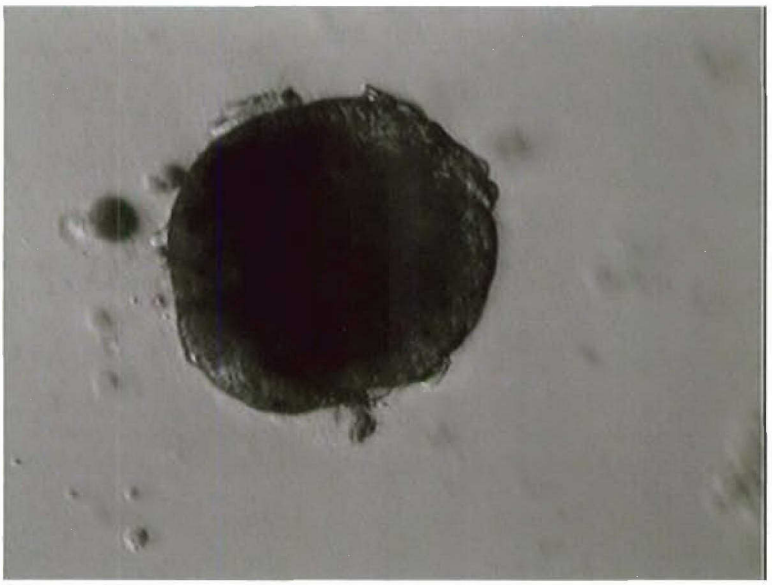


Similarly, the growth of a single dispersed cell from a primary mammosphere treated with DMSO (20X)

is similar to the growth of a single dispersed cell from a primary mammosphere treated with BaP (20X)



and similar to the growth of a single dispersed cell from a primary mammosphere treated with DMBA (20X)



These attempts did not yield "microtumors" as we had proposed and others [9] have reported. While we have not completed immunohistological or morphological analyses of these structures, they appear similar to each other and unrelated to treatment conditions. Studies are currently underway to study the effect of hormone withdrawal on the appearance of these structures. Additional studies are planned to evaluate different doses of DMBA/BaP as well as different combinations of cells from spheres and organoids plated together and separately.

Statement of Work Product: Have reproducible system for enriching stem/progenitor cells and show in replicate studies which cell types are necessary for microtumor formation in vitro following carcinogen exposure and whether the answer is carcinogen specific.

To date we have slight enrichment of stem cells in rat mammary mammospheres, but we believe the enrichment is still insufficient to make this discrimination. Given the paucity of stem cells seen by IHC and by FACS analysis in mammospheres, they are an insufficient source for testing cell type-dependent tumorigenesis. In this proposal we have carefully characterized the cell types in organoids and mammospheres from rat mammary glands. Additional studies are now required to refine the staining requirements for flow-sorting of side populations that exclude Hoechst and then characterizing the response of these progenitor cell populations to carcinogens.

KEY RESEARCH ACCOMPLISHMENTS:

- isolated viable rat mammary epithelial (RME) cells from intact mammary glands
- reproducibly plated single RME (from feshly isolated and viably frozen stocks) in suspension culture on agarose-coated dishes to produce primary non-adherent mammospheres
- reproducibly plated single cells from mammospheres in suspension culture on agarose-coated dishes to produce secondary and tertiary mammospheres demonstrating the self-renewal, regenerating abilities; hallmarks of S/PC
- confirmed that SP cells are present in primary mammospheres by Hoechst-exclusion analysis of both intact spheres and single cells from spheres after dispersement
- yielded FACS analysis of Hoechst-33342 exclusion indicating a population of cells (SP) which effluxes Hoechst dye (termed the arm) as well as a population of non-excluding cells (non-SP) cells termed the head similar to other reports
- confirmed the enrichment of S/PC cells present in mammospheres through formation of new mammospheres from SP cells plated after FACS analysis
- confirmed mammospheres contain S/PC capable of differentiating into lobular and ductal structures when grown on solid substrate (Matrigel)
- immunostaining results with combinations of antibodies to cell-surface proteins indicated that cell populations from organoids and mammopsheres are similar and indistinguishable using this methodology.

CONCLUSIONS:

We have developed a reproducible system for enriching stem progenitor cells isolated from rat mammary glands in which mammospheres are grown from single RME plated in suspension cultures. We have characterized the proportion of specific cell types by cell surface antigen expression in organoids vs. mammospheres. Unlike previous studies in which organoids treated with carcinogens yielded microtumors in vitro, these data suggest that dispersion of the cells following carcinogen exposure prevent formation of this histology. Results from mammosphere and organoids were similar. Additional studies to purify stem cell populations from rat mammary gland must be worked out before we can clearly assess the cell types necessary for carcinogenesis.

REFERENCES

[1] Clarke, R., Anderson, E., Howell, A., and Potten, C. 2003. Regulation of human breast epithelial stem cells. *Cell Prolif*, 36:45-58.

[2] Dontu, G., Abdallah, W.W., Foley, J.M., Jackson, K.W., Clarke, M., Kawamura, M.J., and Wicha, M.S. 2003. In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells, *Genes Dev.* 17:1253-1270.

[3] Heffelfinger, S.C., Yan, M., Gear, R.B., Schneider, J., LaDow, K., and Warshawsky, D. 2004. Inhibition of VEGFR2 prevents DMBA-induced mammary tumor formation, *Lab. Invest.* 84:989-998.

[4] Heffelfinger, S.C., Gear, R.B., Schneider, J., LaDow, K., Yan, M., Lu, F., Pyle, A.L., and Warshawsky, D. 2003. TNP-470 inhibits 7,12-dimethylbenz[a]anthracene-induced mammary tumor formation when administered before the formation of carcinoma in situ but is not additive with tamoxifen, *Lab. Invest.* 83:1001-1011.

[5] Al-Hajj, Wicha, M.S., Benito-Hernandez, A., Morrison, S.J., and Clarke, M.F. 2003. Prospective identification of tumorigenic breast cancer cells, *PNAS*, 100:3983-88.

[6] Welm, B.E., Tepera, S.B., Venezia, T., Graubert, T.A., Rosen, J.M., Goodell, M.A. 2002. Sca-1 (pos) cells in the mouse mammary gland represent an enriched progenitor cell population. *Dev. Biol.* 245:42-48.

[7] Goodell, M.A., Brose, K., Paradis, G., Connor, A.S., Mulligan, R.C. 1996. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo, *J. Exp. Med.* 183: 1797-1806.

[8] Morrison, S.J., Shah, N.M., and Anderson, D.J. 1997. Regulatory mechanisms in stem cell biology, *Cell*, 88:287-298.

[9] Laduca, J.R. and Sinha, D.K. 1993. In vitro carcinogenesis of mammary epithelial cells by N-nitroso-N-methylurea using a collagen gel matrix culture, *In Vitro Cell Dev. Biol.* 29A:789-791.