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Award Number: DAMD17-03-1-0644

TITLE: Characterization of Breast Cancer Stem Cells

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REPORT DATE: August 2004

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;
Distribution Unlimited

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20050302 142

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

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 Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2004	Final (15 Aug 03-15 Jul 04)

4. TITLE AND SUBTITLE
Characterization of Breast Cancer Stem Cells

5. FUNDING NUMBERS
DAMD17-03-1-0644

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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, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Cellular markers which identify putative breast epithelial stem cells have been reported. Attention has focused on the possibility that certain clinical aspects of breast cancer could result from transformed stem cells residing in the malignant tumor. Breast cancer may therefore originate from neoplastic transformation of normal breast epithelial stem cells. These transformed stem cells may exist in the tumor as rare cells with properties which drive multiple aspects of tumorigenesis. The predictions of this model included slower cell cycle progression, greater resistance to DNA damage, increased in vitro invasion, and in vivo tumorigenesis. The application tested the hypothesis that transformed stem cells drive multiple aspects of breast tumorigenesis by functionally characterizing their biological properties. The hypothesis was tested by sorting of putative breast cancer stem cell populations followed by cell cycle analysis, in vitro and in vivo proliferation and invasion assays, and sensitivity to chemotherapy and radiation induced DNA damage. Putative cancer stem cells generally exhibited slower cell cycle progression, greater resistance to DNA damage, and increased tumor formation. However, the degree of stem-like properties of these cells varied widely depending on the line from which they were isolated.

14. SUBJECT TERMS
Biomarkers, cancer biology, endogenous factors

15. NUMBER OF PAGES
11

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Background. Stem cells have been the subject of great interest in recent years because of their unique biological properties: mitotic quiescence, self renewal, and generation of differentiated cell types (Blau et al., 2001). Cellular markers which identify putative breast epithelial stem cells have been reported recently (Gudjonsson et al., 2002). Lately attention has focused on the possibility that certain clinical aspects of cancer could result from transformed stem cells residing within the malignant tumor (Reya et al., 2001). There is evidence that cancer stem cells exist in some hematologic malignancies but the case for transformed stem cells in breast cancer is less clear. Oncogenic mutations could be inherited from transformed epithelial stem cells and thus give rise to diverse tumorigenic populations observed in breast cancer. There are important ramifications for clinical cancer treatment if the growth of breast cancer is dependent on a subset of transformed stem cells. For example, tumor recurrence following traditional antiproliferative therapy may be due to the non-dividing and thus untargeted stem cell compartment repopulating the tumor.

Rationale/Purpose. Breast cancer originates from neoplastic transformation of normal breast epithelial stem cells. These transformed stem cells exist in the tumor as rare cells with properties which drive multiple aspects of tumorigenesis.

Objectives. This application will test the hypothesis that transformed stem cells drive multiple aspects of breast tumorigenesis by functionally characterizing the biological properties of putative breast cancer stem cells.

Methods. The stem cell model of breast cancer makes important predictions that have significant impact on breast cancer biology and treatment. These predictions include: 1) slow cell cycle progression; 2) resistance to anti-proliferative therapy as the result of slow cell cycle progression; and 3) the ability to invade normal tissue and form tumors in experimental models. These predictions will be experimentally tested as described below.

Cell cycle progression and invasive ability of putative breast cancer stem cells. Double immunofluorescence labeling and fluorescence activated cell sorting (FACS) of cultured human breast cancer cells using antibodies to sialomucin and epithelial specific antigen (ESA) revealed sialomucin negative/ESA positive cells comprising 0.5% of the population. This biomarker profile has been proposed for normal breast epithelial stem cells (Gudjonsson et al., 2002). We propose to evaluate cell cycle distribution and proliferation of these two populations by: 1) DNA content analysis using propidium iodide stained cells and FACS; 2) BrdU incorporation; and 3) in vitro proliferation assay. The ability of these two cell populations to invade reconstituted basement membrane will be determined using Matrigel coated Boyden chambers. We predict that the sialomucin negative/ESA positive population will demonstrate slower cell cycle progression and greater ability to invade Matrigel membranes than the sialomucin positive/ESA positive group.

Sensitivity of putative breast cancer stem cells to chemotherapy and radiation induced DNA damage. Since most radiation and chemotherapy treatment protocols target dividing cells, breast cancer stem cells may escape the effects of these therapies due to their relative mitotic quiescence. We propose to evaluate DNA damage in the two sorted breast cancer cell populations (i.e., putative stem vs. non-stem) described above in response to 10 μg/ml etoposide treatment or 10-60 Gy ionizing radiation in vitro. DNA damage will be measured as relative DNA fragmentation in permeabilized cells subjected to single cell gel electrophoresis and analyzed by Komet 5.5 software (Kinetic Imaging, Inc.). Cell death will also be determined by trypan blue exclusion assay. We predict that putative breast cancer stem cells will show less DNA damage and cell death in response to chemotherapy and ionizing radiation.

Tumorigenicity in nude mice. The putative breast cancer stem and non-stem cell populations will be tested for their relative abilities to form tumors in animal models. 10^2 to 10^6 cells from putative stem or non-stem cell populations (control) will be injected into the mammary fat pads of nude mice. The ability of these cells to form detectable tumors will be assessed for up to 6 months. The number and volume of tumors will be assessed in each mouse. Our model predicts that putative breast cancer stem cells will be significantly more efficient in producing tumors in experimental animals.

BODY OF REPORT

This is the progress report of Concept Award DAMD17-03-1-0644 "Characterization of Breast Cancer Stem Cells". Collection and analysis of these data are ongoing; therefore it should be emphasized that the reported results are preliminary and may be modified as additional data are analyzed and statistical analysis performed. We are currently repeating some experiments to obtain photographic records. Double immunofluorescence labeling and fluorescence activated cell sorting (FACS) of cultured human breast cancer cell lines using antibodies to sialomucin and epithelial specific antigen (ESA) revealed a predominant ESA+ population (>99%) in all lines examined (MCF7, T47D, MDA-MB-468, MDA-MB-231, Hs578T, and SKBR3). MCF7 and T47D contained a sialomucin negative population (MUC-) that was much smaller than other cell lines (0.05% and 0.02% respectively). In contrast, the sialomucin negative population in the other four lines was 0.4-0.5%. We also examined additional markers of breast epithelial cell differentiation (Fig. 1). Both populations in all cell lines expressed keratin 18, a marker of simple epithelium. In addition, all cell lines expressed keratin 19, which is expressed by the terminal duct lobular unit. Only SKBR3 expressed detectable levels of smooth muscle actin, a marker of myoepithelial differentiation. We detected phenotypic differences in the ESA+/MUC- population between different breast cancer cell lines. All ESA+/MUC- populations proliferated 10-20% slower than the MUC+ cells; however the MUC- fraction in MCF7 and T47D lines grew at 25-30% slower rates as determined by in vitro proliferation, DNA content analysis, and BrdU incorporation (Fig. 2A, B). We also determined differences in the relative abilities of these populations to invade Matrigel membranes. While the MUC- fraction in the other 4 cell lines were highly invasive in the in vitro assay, this subpopulation in MCF7 and T47D cells were 50-80% less able to penetrate the reconstituted basement membrane (Fig. 3). With respect to DNA damage by etoposide and ionizing radiation, the MUC- population from MCF7 and T47D cells showed 65% less DNA damage in the single cell gel electrophoresis analysis than the sorted fraction from the other 4 cell lines (Fig. 4). However, the MUC- population in these lines was more resistant to DNA damage than the MUC+ fraction. The MCF7 and T47D MUC- population were also 90% less tumorigenic (as measured by tumor volume) in nude mice than the same fraction from the other 4 cell lines (Fig. 5). However, all MUC- populations were more tumorigenic than the MUC+ fractions.

Materials and Methods

Cell Culture and Sorting. Human breast cancer cell lines were cultured in Dulbecco's modified Eagle medium, 10% fetal bovine serum, and 40 μg/ml gentamicin. Double immunofluorescence labeling was performed by incubation of suspended cells with mouse anti-human epithelial specific antigen antibody and goat anti-human sialomucin antibody (20 μl/10^6 cells) at 4^0 C for 2 hours. After extensive washing in PBS, fluorescein conjugated anti-mouse IgG and rhodamine conjugated anti-goat IgG secondary antibodies were incubated with the cells for an additional two hours. After washing in PBS, cells were sorted using a Becton Dickinson flow cytometer.

Western Blot. 25 μg total cellular protein was separated by SDS-PAGE on 10% resolving gels under denaturing and reducing conditions. Separated proteins were electroblotted to PVDF membranes according to manufacturer's recommendations (Roche Molecular Biochemicals). Blots were incubated with antibodies to human smooth muscle actin, keratin 18, or keratin 19 (Sigma) for 16 hours at 4^0 C. After washing in Tris buffered saline containing 0.1% Tween 20 (TBST, pH 7.4), blots were incubated for 30 minutes at room temperature with anti-IgG secondary antibody conjugated to horseradish peroxidase. Following extensive washing in TBST, bands were visualized by the enhanced chemiluminescence method (Roche Molecular Biochemicals).

Cell Growth and Bromodeoxyuridine Incorporation Analysis. 3 x 10^4 sorted cells were cultured on tissue culture plastic dishes for up to six days. At two day intervals, cultures were trypsinized and counted using a hemacytometer. Duplicate cultures were incubated with 10 μM BrdU for 1 hour. After washing in PBS, cells were fixed in 70% ethanol, 50 mM glycine (pH 2) for 30 minutes at -20^0 C. After extensive washing in PBS, cells were incubated with mouse anti-BrdU primary antibody at 37^0 C for 30 minutes (Roche Molecular Biochemicals). After washing in PBS, cells were incubated with anti-mouse IgG secondary antibody conjugated to fluorescein at 37^0 C for 30 minutes. Following extensive washing in PBS, BrdU positive cells were visualized by fluorescence microscopy. The number of positive cells was expressed as a percentage of total cells counted in ten randomly selected high power fields.

In Vitro Invasion Assays. 2 x 10^5 sorted cells were plated into modified Boyden chambers coated with Matrigel reconstituted basement membrane (Becton Dickinson) for 24 hours. Cells that were able to invade the semi-permeable membrane were fixed in methanol, stained with hematoxylin, and counted.

DNA Damage Analysis. 3 x 10^4 sorted cells were plated onto tissue culture plastic dishes for 24 hours before being treated with 10 μg/ml etoposide for 24 hours or exposed to 20 Gy ionizing radiation. Single cell gel electrophoresis then was performed following the manufacturer's instructions (Kinetic Imaging). Quantitation of DNA damage (relative tail moment) was performed using the manufacturer's image analysis software.

In Vivo Tumorigenesis. 2 x 10^5 sorted cells were injected subcutaneously into the mammary fat pads of nude mice. After one month, the fat pads were dissected and any tumors that developed were measured. Tumor volume was calculated as the product of the measurements in three axes.

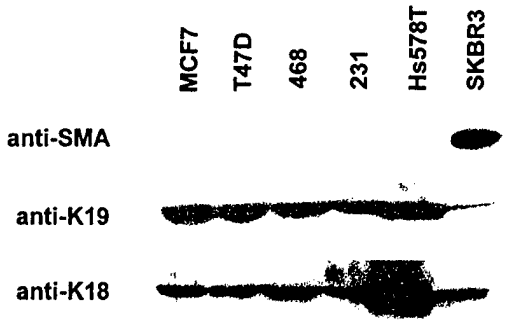
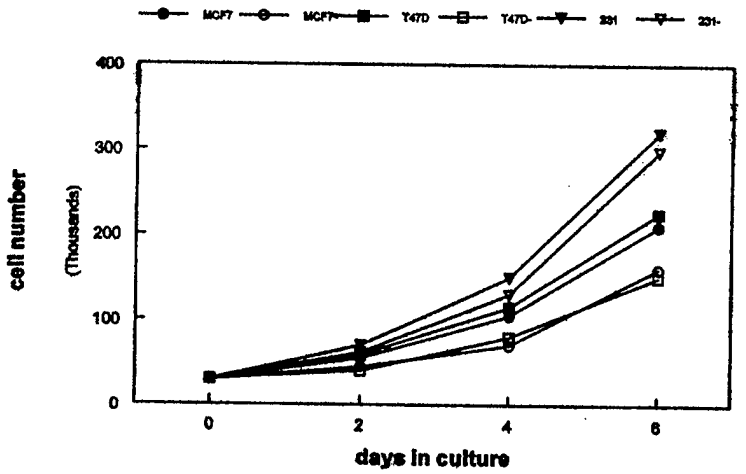


Fig. 1. Expression of differentiation markers in human breast cancer cell lines. Six breast cancer cell lines were examined by western blot using antibodies to the myoepithelial marker smooth muscle actin (SMA), the terminal ductal marker keratin 19 (K19), and the simple epithelial marker keratin 18 (K18). Representative blots are shown.

A



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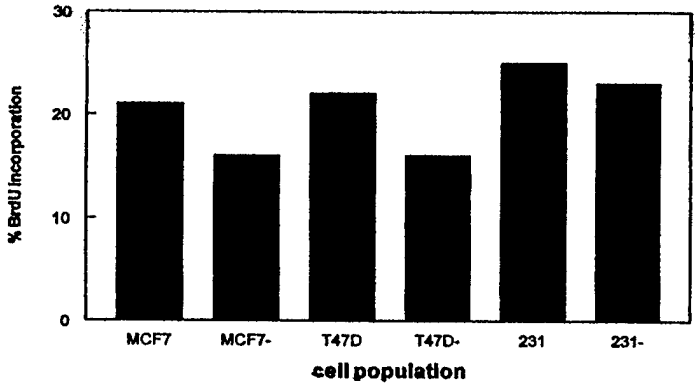

Fig. 2. Decreased proliferation of MUC- cells in human breast cancer cell lines. Shown above are data from MCF7, T47D, and MDA-MB-231 lines. (A) MUC negative (-) and MUC+ cell sorts were cultured for up to 6 days. At two day intervals cells were counted using a hemacytometer. (B) Sorted fractions were labeled with BrdU for 1 hour prior to visualization of BrdU positive cells using anti-BrdU antibody. Data is reported as percentage of cells that incorporated BrdU.

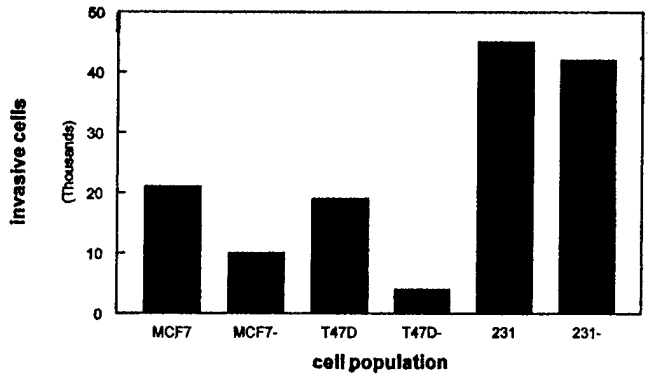

Fig. 3. Decreased invasive ability of MUC negative (-) population in MCF7 and T47D cell lines. MUC+ and MUC- cells from T47D, MCF7, and MDA-MB-231 lines were plated into Matrigel invasion chambers for 24 hours. Cells which invaded through the reconstituted basement membrane were fixed, stained, and counted.

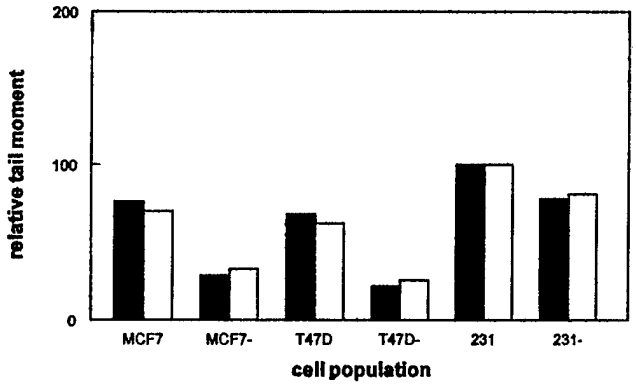

Fig. 4. Decreased DNA damage in the MUC negative (-) fraction after exposure to 10 μg/ml etoposide (filled bars) or 20 Gy ionizing radiation (open bars). MUC+ and MUC- cells from T47D, MCF7, and MDA-MB-231 lines were plated on tissue culture plastic dishes and subjected individually to one of the DNA damage protocols. Cells were then subjected to single cell gel electrophoresis. The extent of DNA damage (relative tail moment) was calculated based on signal intensity and migration.

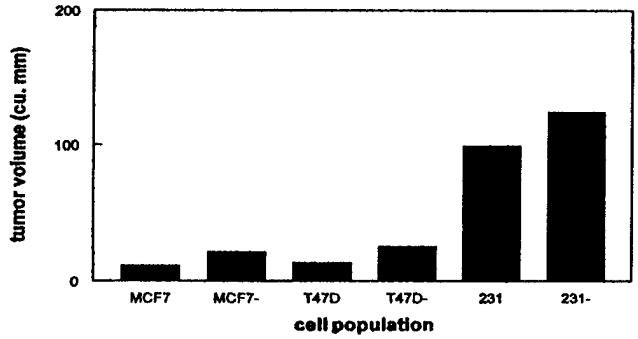

Fig. 5. The MUC- fraction (-) is more tumorigenic than the MUC+ fraction in human breast cancer cell lines. MUC+ and MUC- cells from T47D, MCF7, and MDA-MB-231 lines were injected into the mammary fat pads of nude mice. The tumor volume after one month was calculated from measurement of dissected tumors.

KEY RESEARCH ACCOMPLISHMENTS

Task 1

1. All ESA+/MUC- populations proliferated 10-20% slower than the MUC+ cells; however the MUC- fraction in MCF7 and T47D lines grew at 25-30% slower rates.
2. This MUC- fraction from MCF7 and T47D but not other lines were able to form mammospheres in suspension culture.
3. The MUC- fraction in the other 4 cell lines was highly invasive in the in vitro assay; however, this subpopulation in MCF7 and T47D cells were 50-80% less able to penetrate the Matrigel membrane.

Task 2

1. The MUC- population from MCF7 and T47D cells showed 65% less DNA damage in the single cell gel electrophoresis analysis than the sorted fraction from the other 4 cell lines.
2. The MUC- population in all lines was more resistant to DNA damage than the MUC+ fraction.

Task 3

1. The MCF7 and T47D MUC- population were 90% less tumorigenic when injected into the mammary fat pads of nude mice than the same fraction from the other 4 cell lines.
2. The MUC- populations were more tumorigenic than the MUC+ fractions.

REPORTABLE OUTCOMES

Not applicable

CONCLUSIONS

In the first ten months of the funded application we determined that MUC- cells exist in human breast cancer cell lines whose phenotypic properties are different than those of the MUC+ population. However, the numbers of these cells vary widely and their properties are somewhat variable depending on the line from which they were derived. The cells also expressed additional markers of epithelial and myoepithelial differentiation. These cells were difficult to maintain for extended time periods in culture due to rapid generation of MUC+ progeny. We therefore used short term assays to characterize these populations. All ESA+/MUC- populations proliferated slower than the MUC+ cells; however the MUC- fraction in MCF7 and T47D lines grew even slower than those found in other lines. This cell fraction from MCF7 and T47D but not other lines were able to aggregate and form mammospheres in suspension culture, an ability that has been ascribed to putative breast epithelial stem cells (Dontu et al., 2003). While the MUC- fraction in the other 4 cell lines were highly invasive in the in vitro assay, this subpopulation in MCF7 and T47D cells were less able to penetrate the reconstituted basement membrane. The MUC- population from MCF7 and T47D cells showed less DNA damage in the single cell gel electrophoresis analysis than the sorted fraction from the other 4 cell lines. However, the MUC- population in these lines was more resistant generally to DNA damage than the MUC+ fraction. The MCF7 and T47D MUC- population were also less tumorigenic when injected into the mammary fat pads of nude mice than the same fraction from the other 4 cell lines. However, all MUC- populations were more tumorigenic than the MUC+ fractions. Therefore the MUC- fraction from different lines demonstrated putative stem cell characteristics to variable extents. Despite the use of short term assays, it was not possible to rule out completely the contribution of the MUC+ progeny of these cells to the observed phenotypes. In future experiments it will be important to determine how to maintain these cells in the MUC- state so that long term observations may be undertaken. The factors which determine the degree of stem-like character in a mixed population of breast cancer cells will also need to be determined. The ability to isolate these cell fractions should lead to characterization of additional markers which may provide clues to their proliferation, differentiation, invasion, and tumorigenicity.

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