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TITLE: Identification of Stem Cells in a Novel Human Mammary Epithelial Culture (HMEC) System that Reproducibly Demonstrates Ductal Organotypic Architecture in 3 Weeks

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**14. ABSTRACT:** Our laboratory has published a novel culture system for Human Mammary Epithelial Cells (HMEC), both normal and malignant. This system allows for unusually long-term (3 months or longer) establishment of normal primary cultures that begin as three-dimensional "mammospheres," which are structures made up of 40-100 epithelial cells. These mammospheres subsequently differentiate into complex organotypic branching ducts and lobules that demonstrate Epithelial Specific Antibody (ESA) staining, lumen, polarized nuclei, desmosomes along the lateral surfaces of the cells, and microvilli on the apical surfaces We hypothesize that since we have demonstrated de novo formation of multicellular organotypic epithelial ductal and lobular structures, that our cultures must contain multipotent stem cells, possibly because of the use of our novel tissue culture medium and the use of matrigel. We will identify the stem cell component of our HME cultures and determine the percentage of cells that have this profile. We will isolate these putative stem cells and reintroduce them after labeling, into our culture system to determine their ability to contribute to multiple lineages under the juxtacrine and paracrine influences of the other cells present. We will dentify and isolate putative mammary stem cells using Hoechst exclusion from our primary cultures before they have actually differentiated ductal structures (pre-14 days in culture). Determine whether these flow sorted cells match

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## Introduction:

a. Background: Our laboratory has developed and published a novel culture system for Human Mammary Epithelial Cells (HMEC), both normal and malignant. This system allows for unusually long-term (3 months or longer) establishment of normal primary cultures that begin as three-dimensional "mammospheres," which are structures made up of 40-100 epithelial cells. These mammospheres subsequently differentiate into complex organotypic branching ducts and lobules that demonstrate Epithelial Specific Antibody (ESA) staining, lumen, polarized nuclei, desmosomes along the lateral surfaces of the cells, and microvilli on the apical surfaces. Type 2 lobules are often present at the ends or junction points of these ductal structures. Tumor cells, in contrast, manifest a continuum of behaviors in our culture system, but completely lack the ability to form these collaborative epithelial architectures. We have produced time-lapse digital movies of tumor and normal epithelial cell behavior by capturing a digital image every 10 minutes for 12-48 hours using a Hamamatsu digital camera integrated into a Macintosh G4 computer outfitted with QED imaging systems. Cultures are kept viable using a Bioptechs FCS2 environmentally controlled chamber. The movies demonstrate that the mammospheres form after initial plating of the cells and that the ducts form via cell migration, proliferation and finally cavitation of the epithelial columns. Although elegant in nature, none of the published studies to date demonstrate the formation of **ductal structures from human breast tissue**, which we have reproducibly and uniquely demonstrated.

b. Rationale/Purpose: We hypothesize that since we have demonstrated de novo formation of multicellular organotypic epithelial ductal and lobular structures, that our cultures must contain multipotent stem cells, possibly because of the use of our novel tissue culture medium (which does not contain lactation-inducing hormones), the use of matrigel.

c. Objectives: We will identify the stem cell component of our HME cultures and determine the percentage of cells that have this profile. We will isolate these putative stem cells and reintroduce them after labeling, into our culture system to determine their ability to contribute to multiple lineages under the juxtacrine and paracrine influences of the other cells present. Future studies designed to expose these stem cells to mutagenic agents will be performed to understand the consequences of stem cell transformation as one mechanism of breast cancer etiology.

d. Methods: A. Identify and isolate putative mammary stem cells using Hoechst exclusion from our primary cultures before they have actually differentiated ductal structures (pre-14 days in culture).

B. Determine whether these flow sorted cells match the profile of human mammary stem cells currently in the literature: $ESA^+$, $MUC^-$, $\alpha_6$ $integrin^+$. Previous studies have identified bipotent progenitor cells in primary breast epithelial cell cultures using stains for $ESA^+$, $MUC^-$ cells, and $\alpha_6$ $integrin^+$. Through the use of immunohistochemistry and confocal microscopy with these cell markers as well as others such as anti-smooth muscle actin, and cytokeratins CK14 and CK18, we will identify our flow-sorted cells.

C. Transduce flow sorted stem cell population with a Green Fluorescent Protein (GFP) expressing viral construct and reintroduce these cells at the same time point of differentiation into primary culture system in order to determine the functionality of these

cells in the differentiation of organotypic architecture. In effect, we will perform lineage tracing with the expression of GFP. We will document this lineage tracing by creating live cell digital microscopy movies under fluorescence optics.
e. Relevance: **Scientifically** our study will show that there are several methods for maintaining HME stem cells in vitro. **Clinically this study on stem cells will allow many laboratories to test the long held hypothesis that some breast cancers are derived from the accumulated somatic mutation of an adult mammary stem cell**. The consequences of such an in vitro malignant transformation can now be manifested in our HME system.

Papers in the literature now assert that unusual ductal hyperplasia of the breast is a committed stem (progenitor) cell distinct from atypical ductal hyperplasia and ductal carcinoma in situ, but they need further validation with firm HME stem cell markers. The transformation of stem cells may help explain the heterogeneity of this disease at several levels.

## Body (based upon the statement of work):

*1. Identify and isolate putative mammary stem cells using Hoechst exclusion from our in vitro cultures before they have actually differentiated ductal structures (pre-14 days in culture).*

*2. Determine whether these flow sorted cells match the profile of human mammary stem cells currently in the literature: ESA+, MUC-, $\alpha$6 integrin+. Previous studies have identified bipotent progenitor cells in primary breast epithelial cell cultures using stains for ESA+, MUC- cells, and $\alpha$6 integrin+. Through the use of immunohistochemistry and confocal microscopy with these cell markers as well as others such as anti-smooth muscle actin, and cytokeratins CK14 and CK18, we will identify our flow-sorted cells.*

We have developed a novel tissue engineering system for culture of Human Mammary Epithelial Cells (**HMEC**), both normal and malignant. This system allows for unusually long-term (>3 months) establishment of normal primary cultures that begin as 3-dimensional "mammospheres," (**1- 4**) which are structures made up of 40-100 epithelial cells (Fig. 1). These mammospheres differentiate into complex organotypic branching ducts and lobules (Fig. 2) that demonstrate Epithelial Specific Antigen (ESA) staining, cytokeratin-18 and -19 staining, lumen, polarized nuclei, desmosomes along the lateral cell surfaces, and microvilli on apical surfaces (**2**). Type 2 lobules are often present at the ends or junction points of these ductal structures (Figs. 1, 3). Tumor cells, in contrast, show a continuum of behaviors in our culture system (**3**). Although elegant in nature, none of the published studies demonstrate the formation of ductal structures from human breast tissue, which we have reproducibly and uniquely demonstrated.

Ductal structures often begin as linear columns of epithelial cells (pre-ductal linearization). Cavitation of these structures occurs within a few days and is visible as the acquisition of a hollow lumen using bright field microscopy (Figs.1, 3A) or by chemically staining the cell nuclei (**2**). The ducts are linear or branching. Later, formation of lobules occurs, either within or at the end of the duct (Fig. 3). The

presence of either type of lobule represents a more advanced state of differentiation. Our analysis of the preliminary data involves the development of luminal ductal structures without the inclusion of the pre-ductal linearization (earlier) or formation of lobules (later) (Fig.1).

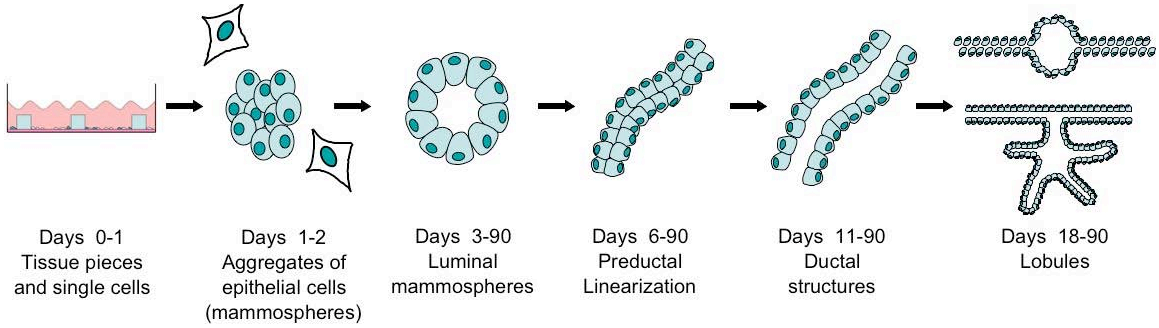


**Fig. 1.** Schematic of the Latimer human mammary tissue engineering system outcomes. This timeline shows the progressive differentiation manifested by non-diseased breast reduction mammosplasties placed into primary culture. Luminal ductal structures were the evaluated endpoint of the data shown in this proposal**. Both human ductal differentiation and the formation of terminal ductal lobular units in vitro, are unprecedented in the literature.**

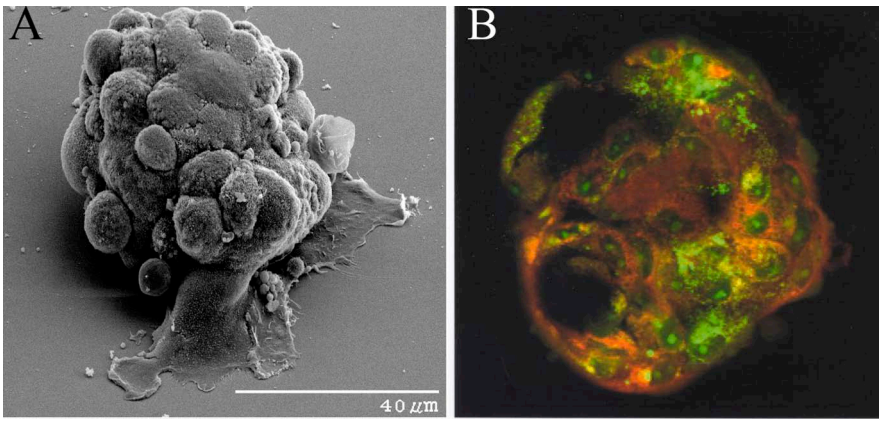


**Fig. 2. (A)** Scanning electron micrograph (SEM) of a normal epithelial mammosphere after 2 days in culture. These cells remain in close contact via desmosomes, and retain normal epithelial tissue architecture and polarity. **( B)** Confocal microscopy image of a mammosphere, showing a view of **the luminal interior of the mammosphere**. Nucleoli are stained with acridine orange (green) and Mitochondria are stained with mitrotracker red (red). The depth of the mammosphere is **66 μm** (2).

We have established primary HMEC cultures from 37/37 breast reduction mammoplasty tissues. These included tissue from: 28 pre-menopausal patients, 5 post-menopausal patients, and 4 peri-menopausal (ages 45-55) patients. The tissues were all shown to be within the range of normal histologies including hyperplasia and fibrocystic disease. No tissue was cultured that contained atypia. We collected these tissues Magee-Womens Hospital where insured women patients are predominantly middle or upper class. Six of the 37 samples were from AA women (16%).

All of the HMEC cultures formed mammospheres; however, less than 50% of those cultured past 11 days formed ductal structures. In an attempt to understand the possible intrinsic factors correlated with the ability to differentiate in culture, we obtained

clinical and basic demographic and medical information on all of the anonymized subjects under our **IRB (#0504117).**

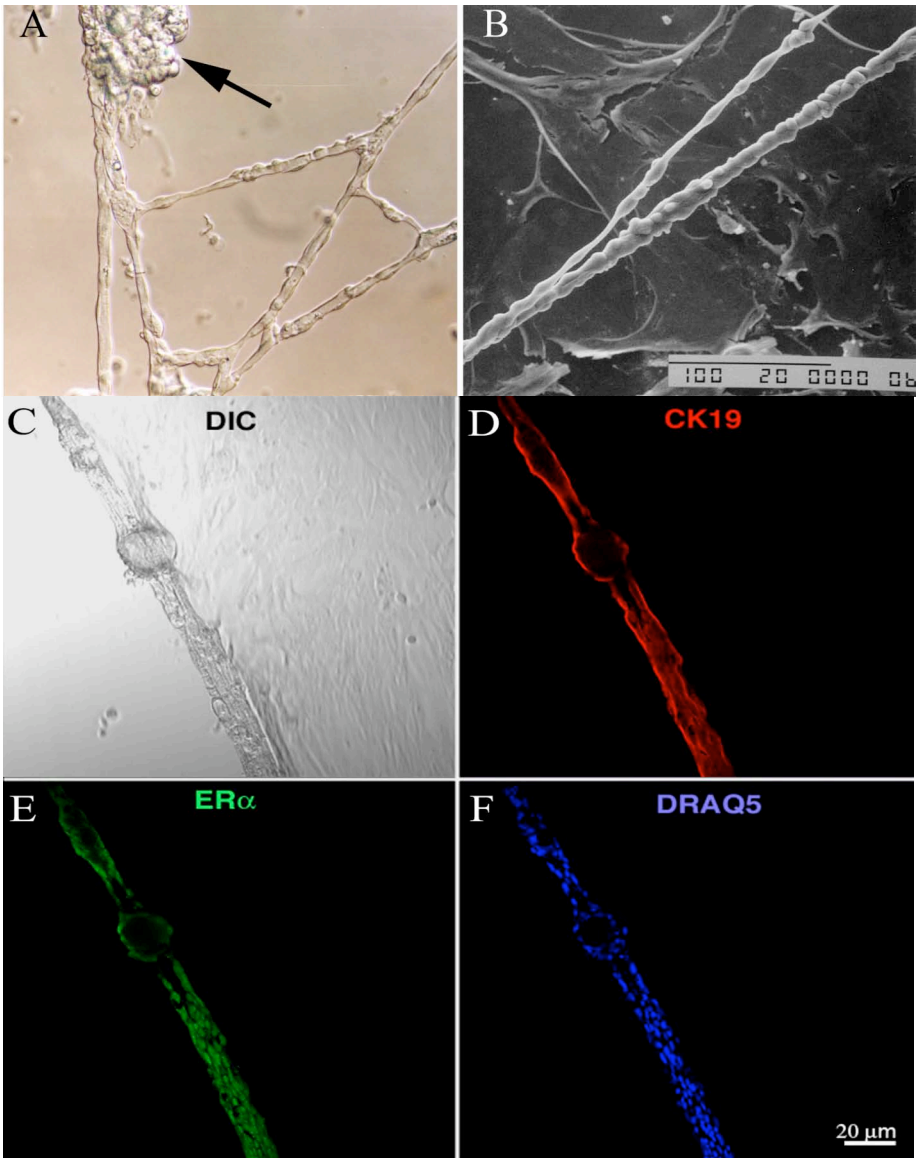


**Fig. 3. (A.)** Branching ductal structures in 15-day-old primary cultures of A. normal breast reduction epithelium (Differential Interference Contrast [DIC] optics, 200X magnification). **(B.)** SEM of similar branching luminal ducts that shows typical suspension about 30 µm above stromal fibroblasts in the dish. Calibration line corresponds to 100 µm. These ductal structures have been shown to contain luminal epithelial cells with apical microvilli by Transmission Electron Microscopy (data not shown) (28). The large mass of cells at the top **(arrow) is a lobule**, which represents a further advanced state of differentiation. (**C, D, E, F**) show a fixed duct containing an **intraductal lobule** under **DIC optics (C)**, and under epifluorecence with **CK-19 (red cytoplasmic) (D),** Estrogen Receptorą **ER α (Green nuclear) (E),** and nonspecific nuclear chemical stain **DRAQ5 (blue) (F**).

<u>Cell lines Established in the Latimer Laboratory</u>

Our laboratory has developed a novel tissue engineering system for **H**uman **M**ammary **E**pithelial **C**ells (**HMEC**), both normal and malignant. Normal cells reiterate

the ductal structure of the breast and contain epithelial and stromal cell types that support each other's existence. Tumor cells, in contrast, show a continuum of behaviors in our culture system although they completely lack the ability to form these collaborative epithelial architectures. None of the published studies demonstrate the formation of **ductal structures from human breast tissue**, which we have reproducibly and uniquely demonstrated (Fig. 3). From these robust primary cultures we have generated 109 explants (<13 passages) and cell lines (>13 passages) without the use of exogenous transforming agents. These cultures have been generated from: breast tumors, non-tumor adjacent tissue (20 matched isogenic pairs) and non-diseased breast reduction mammoplasty tissues derived from Magee Womens Hospital (MWH). Our recent focus has been to characterize several of these lines in great depth in order to publish and disseminate them. Approximately 10 of the best characterized of these cell lines are in the hands of collaborators who have verified that the CGH profiles and the karyotypes of these cells are consistent with the tissue of origin.

Our laboratory is in a unique position to perform this study because of our ability to culture human breast tissue and tumors at higher success rates for establishing explants **(100% for normal breast tissue and 85% for breast tumors)** than those currently shown by the **literature (< 2% for non-diseased breast tissue and 15% for tumors** [**5**]).

Three cell lines are shown in our preliminary data: **JL BRL-23** and **JL BRL-24 and JL BTL-12**. JL BRL-23 and -24 are the 23rd and 24th cell lines created by our laboratory from **non-diseased breast mammoplasty** cultures. JL BRL-23 is derived from a white pre-menopausal subject and JL BRL-24 is derived from an African American pre-menopausal subject**. Both of these lines have demonstrated normal karyotypes at 20 passages** (data not shown). Microarray data show that these lines are very distinct from the commercially available stage IV pleural effusion breast tumor cell lines MCF-7, MDA MB231 (Fig. 4) but contain gene expression consistent with a mixed population of epithelial, stromal and myoepthelial cells (data not shown).

**JL BTL-12** is the 12th of 40 **tumor derived** cell lines we have generated. It is derived from **a stage III chemotherapy naive, ER + tumor** derived from a white patient at MWH. **JL BTL-12 has a very abnormal karyotype with the presence of many marker chromosomes** at 20 passages (data not shown). The microarray profile of JL BTL-12 is similar to that seen for MDA MB231 (Fig. 4).

Stem Cell Preliminary Data

Since we are able to continuously passage and culture the breast reduction cells lines shown in this proposal (as well as others) with a stable and normal karyotype, we hypothesize that **we are enriching for a stem cell compartment**. In addition, the karyotype of the JL BTL-12 cell line appears to be stable after continuous passaging. Our preliminary immunohistochemical data (Figs. 5-8) and particularly our flow sorting data (Figs. 9-11) support this assertion.

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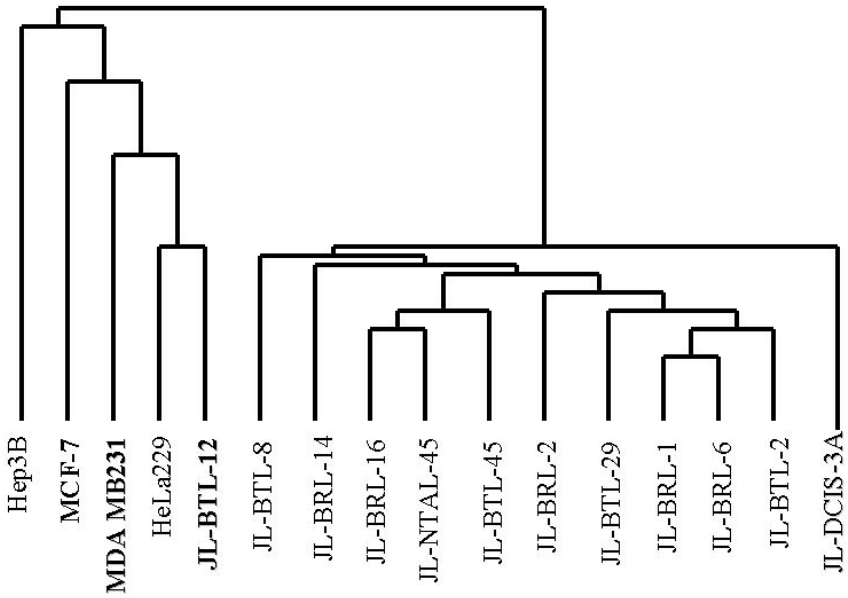


**Fig. 4** Dendrogram of several of Dr. Latimer's established cell lines including JL BTL-12 (stage III tumor derived) which shares gene expression with several highly transformed stage IV commercially available cell lines and is quite dissimilar with early stage BTL cell lines and non-diseased Breast Reduction cell lines (BRL) established by the same culture regimen.

The stem cell markers listed in several prominent and recent papers (**6-9**) include CD24 -, CD44 +, ESA (Epithelial Specific Antigen) +, CD227 (Muc1) -, CD49f ($\alpha_6$ integrin)+ and we utilized all of these markers in our preliminary studies. The cell surface markers (CD24, CD44, CD227, CD49f) have all been utilized for live cell flow cytometry and the cytoplasmic markers cyokeratin-18, ESA have been shown in fixed immunohistochemical cells

Immunohistochemical Staining

Dr. Beer Stolz and Dr. Latimer's laboratories performed staining for a number of proteins identified with stem cells or breast stem cells. Early passage breast reduction cell explants (and in some cases later passage lines) retain the ability to form attached mammospheres (Fig. 5). These mammospheres stain for ESA, CK-18 and CK-19 but they do not stain with the myoepithelial marker, CK-14. Upon passaging and not allowing enough time for mammospheres to form in vitro, we see a thick growth of cells that one cannot describe as a "monolayer" since it is obviously more complex than that. A large percentage of these reduction mammoplasty cells stain with ESA (Fig. 6) suggesting that they are epithelial cells or that some of them may be stem cells.

Staining of JL BTL-12 and some of our other tumor derived cell explants and cultures demonstrates that the majority of these cells stain with CK-18 and -19 suggesting that these cells arose from an epithelial cells (Fig. 7). Interestingly ESA staining shows relatively few cells in the range of 3-5% of the total culture (Fig. 8) which is consistent with what others have found to be the stem cell compartment of breast tumors (**6-9**).

Flow Cytometry Data

Our laboratory was able to develop significant data use cell surface markers rather than Hoecht dye exclusion and so we pursued this aim using these markers.

Cells were disaggregated, counted and $1x10^6$ cells were transferred per 5ml tube and incubated in 50 µl of the appropriate dilution of antibodies in HBSS containing 2%

FBS (fetal bovine serum). Cells were incubated for an hour on ice, washed in HBSS containing 2% FBS. Cells were re-suspended in 0.5ml of HBSS/2%FBS that contained the viability dye propidium iodide (PI, 10µg/ml) to detect dead cells. The antibodies used were CD227-FITC (fluorescein), CD49f-PE (phycoerythrin), CD24/anti-mouse-bio/Streptavidin Cy7-APC, CD44-APC, CD81 (TAPA-1)-APC, CD90-APC-Cy7. All antibodies were purchased from BD Bioscience. Dead cells were eliminated using the viability dye PI. Flow cytometry was performed on a FACSVantage SE DiVa (Becton Dickinson) in Dr. Eric Lagasse's laboratory.

In the normal cultures (BRL-23, -24) two populations of cells exist as shown by auto-fluorescence (Figs. 9C upper, 9C lower). All the cells were subjected to 4 color flow sorting using antibodies against CD49f ($\alpha_6$ integrin), CD227 (Muc1), CD24 and CD44. The cells derived from normal breasts, JL BRL-23, -24 show a staining pattern for these 4 markers consistent with that of published adult breast stem cells (CD24 -, CD44 +,

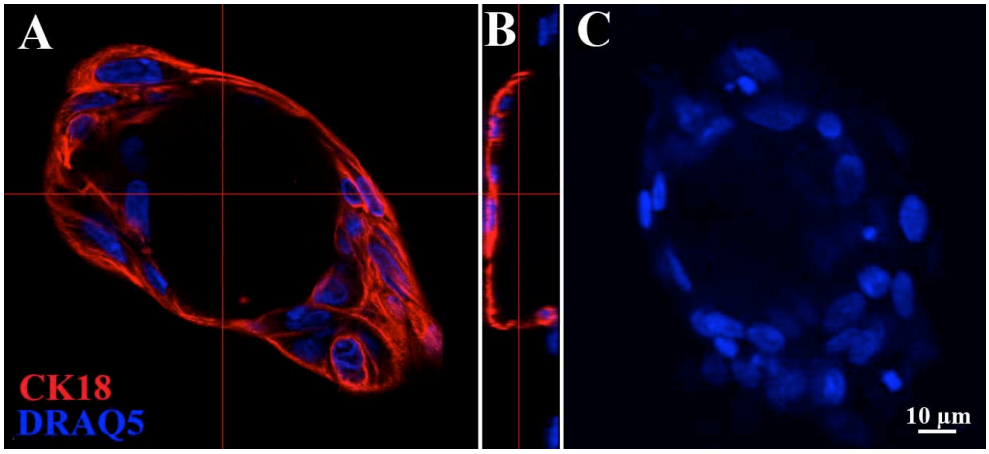


**Fig. 5. (A)** JL BRL-23 (Non-diseased Breast Reduction Line) in early passage retains the ability to form luminal epithelial structures in vitro. CK 18 staining shows the epithelial nature of these cells is retained in culture. This explant culture has been fixed and immunohistochemical stained with cytokeratin 18 (Texas Red). Nuclei are counterstained with the chemical stain DRAQ5. **(B)** Confocal X/Z axis view shows that the stain is visible in all 3 dimensions. **(C)** Non-immune control shows the total absence of CK 18 Texas Red staining.

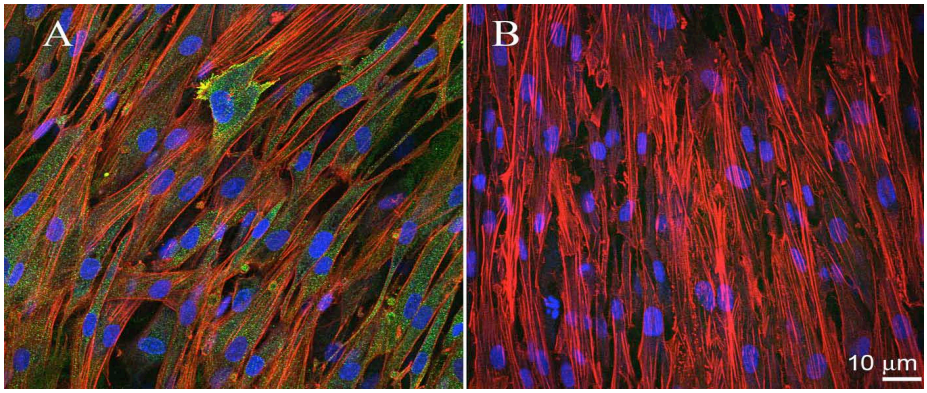


**Fig. 6. (A)** Breast Reduction line 23 (JL BRL-23) after many passages in culture retains staining with FITC labeled **Epithelial Specific Antigen (ESA) (green)**. Multiple cell types are retained in our BRL cultures as can be seen here and in the flow sorting auto-fluorescence data. ESA is labeled with FITC. Nuclei are counterstained with the chemical stain DRAQ5 and the cytoplasm is chemically counterstained with rhodamine**.** **(B)** Non-immune control shows the total absence of ESA-FITC staining.

CD227-, CD49f +) and approximately 100% of the cells that were CD44+ and CD24- were also CD227 - and CD49f +. **These data would suggest that our culture system might be enriched or enriching for self-renewing stem cells**. Both of these breast reduction cells lines show great similarity under flow including the CD90 and CD81 profiles that show great positivity for both markers in these cells (Fig. 9E upper, lower) relative to the non-immune controls (Fig. 9A, C upper, lower).

In contrast, the tumor cell line BTL-12 (derived from a stage III ER+ breast tumor), showed 36.4% were CD49f +, 94.7% were CD227 negative and 14% were CD44+ and 4% were CD24-. This means that nearly all of the tumor cells were positive for CD24, a very marked difference between these cells and the BRL cell lines tested. In addition a very different profile for CD90 and CD81 was shown for JL BTL-12 (Fig. 10E). This profile was exactly the opposite seen for the normal breast reduction cell lines (Fig. 9E upper and lower).

**The fact that we see such different profiles in the tumor cell line in contrast with the 2 breast reduction cell lines shows that although all of these lines have been cultured in identical ways with matrigel and with our very rich MWRI medium, we are capturing cell lines with different karyotypes, cytogenetic profiles, and flow sorting profiles**. Our system of culture is allowing for heterogeneity to be manifested in these different cell populations.

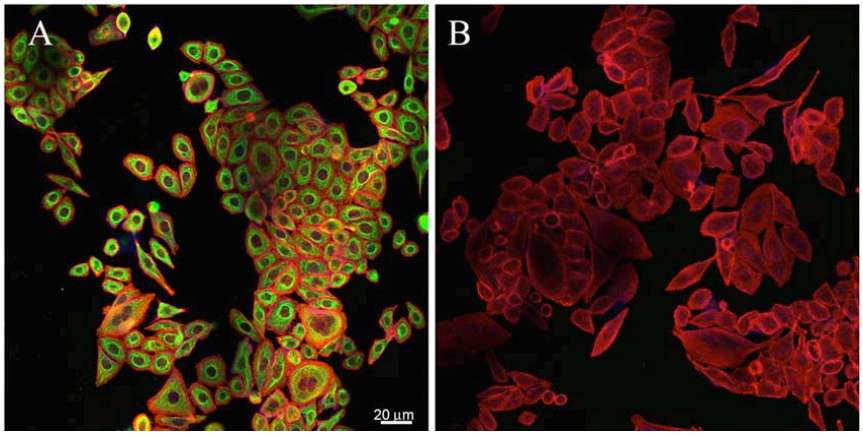


**Fig. 7. (A) JL BTL-12 stage III breast tumor cell line** stained with FITC labeled **Cytokeratin 18 (green)**. All of the cells stain with CK 18 that is **confirmation of their luminal epithelial origin**. Cells are chemically counter stained with a non-specific rhodamine**. (B**) Non-immune controls are shown to be entirely devoid of FITC-CK 18. This tumor cell line has a very epithelial morphology although heterogeneity exists as shown by the flow sorting data.

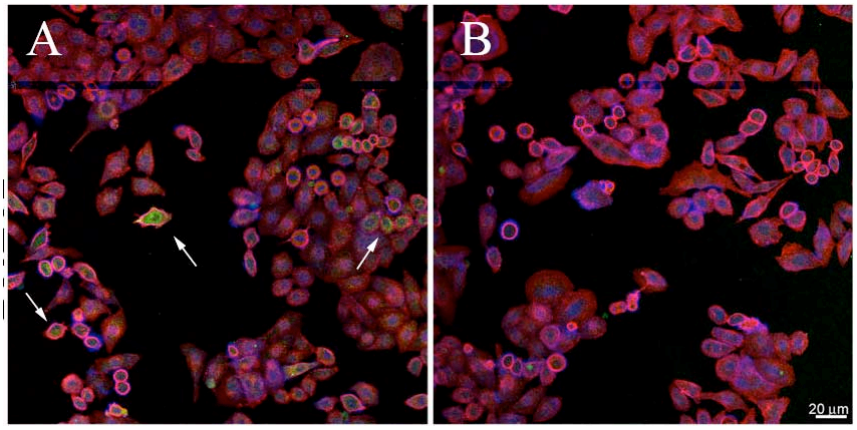


**Fig. 8. (A)** JL BTL-12 Stained with FITC labeled Epithelial Specific Antigen antibody (**ESA**) (**GREEN**). A small percentage of these tumor cells stain with **ESA (labeled with white arrows).** ESA staining has been shown to be consistent with tumor stem cells. Cells are counter stained with a non-specific rhodamine counter stain and a blue DRAQ5 nuclear stain. **(B**) Non-immune controls are shown. Scale bars shown represent 20 µm.

We can detect multiple populations within each of these cell lines by virtue of the auto-fluorescent differences or detected by shifts in populations after staining with specific antibodies. This may mean that we are retaining populations that may be bi-potent and self-renewing. Xenograft studies can confirm this and although those experiments are planned for the future, they are beyond the scope of this grant proposal.

Both breast normal cell lines (BRL23 and BRL24) had similar cell surface markers (CD44+CD24-CD227-CD81+CD90+ and a fraction of cells CD49f+weakly positive) while breast cancer cell line BTL12 had a distinct expression of the same markers (CD44+CD24+CD227-and +CD81+CD90).

Since much of the work in stem cells in breast has been done on breast tumors, we further analyzed our JLBTL-12 flow cytometry data (Fig. 11). Gating of the cells that are CD44+/CD24- (**34**) in the JL BTL-12 cultures shows 0.94% of this entire population fits this profile. Nearly 100% of these cells (all but 4) also fit the profile consisting of $\alpha_6$ integrin (CD49f)+/ Muc1 (CD227)-. This indicates that we very likely have stem cells in this cell line and the means to evaluate stem cells in our JL BTL-12 cell line**. It further means that our in vitro system has the capacity to maintain these stem cells even after 28 passages (the passage number shown in this grant proposal)**. Similar analysis of the 2 non-diseased breast cell lines showed that all of the CD24-/CD44+ cells showed negativity for Muc1 (CD227) and positivity for integrin $\alpha_6$ (CD49f)+.

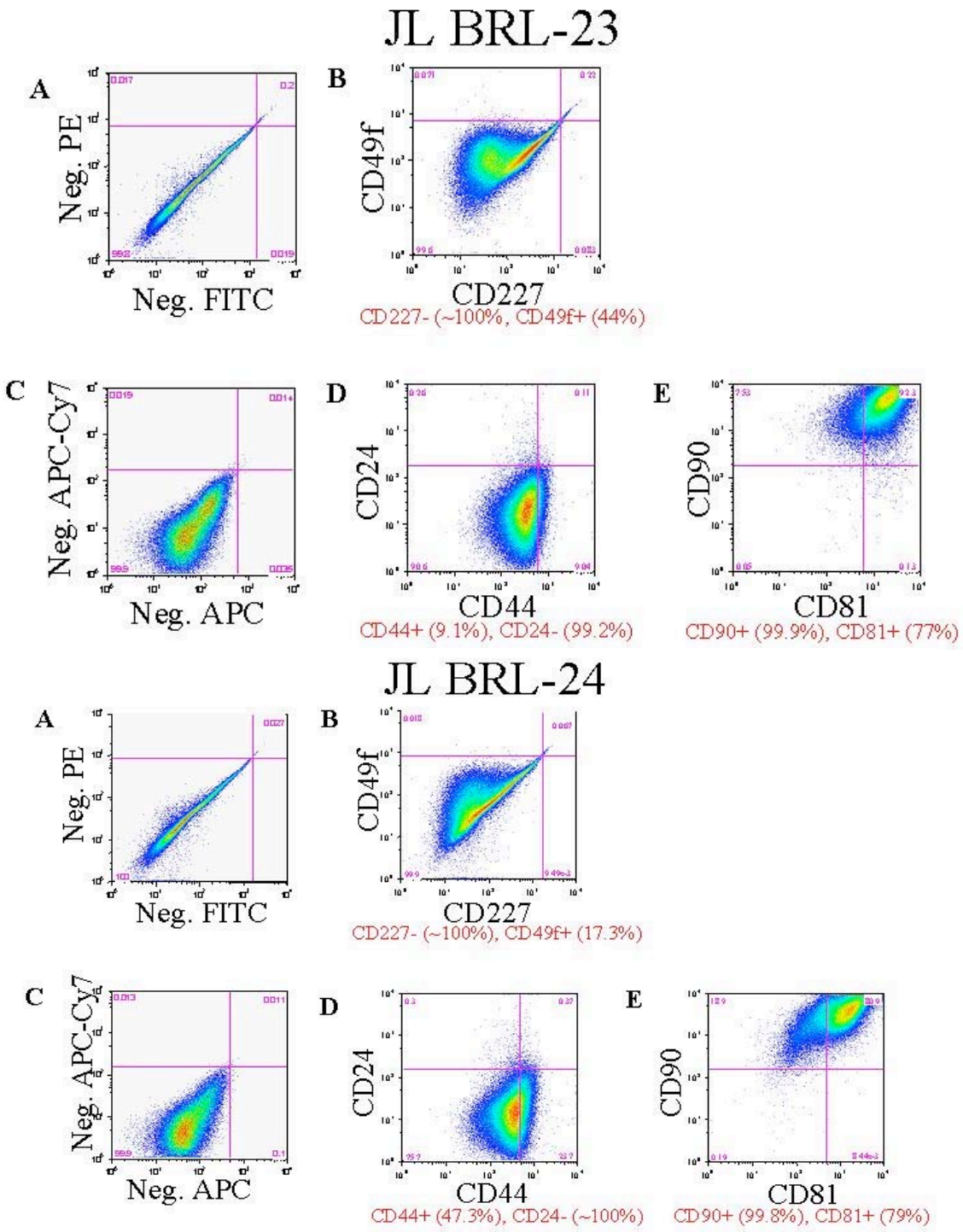


**Fig. 9. Analysis** by flow cytometry of the 23rd and 24th Breast Reduction Lines established in the Latimer Lab. In the normal cultures (BRL-23, -24) two populations of cells exist as shown by auto fluorescence. Cells were stained with CD227 (Muc-1)-FITC, CD49f ($\alpha$-$_6$ integrin)-PE, CD44-APC and CD24/anti-mouse-bio/Streptavidin Cy7-APC or CD81 (TAPA-1)-APC and CD90-bio (Thy-1)/Streptavidin Cy7-APC). Dot plots represent the gated viable cells (PI- cells).

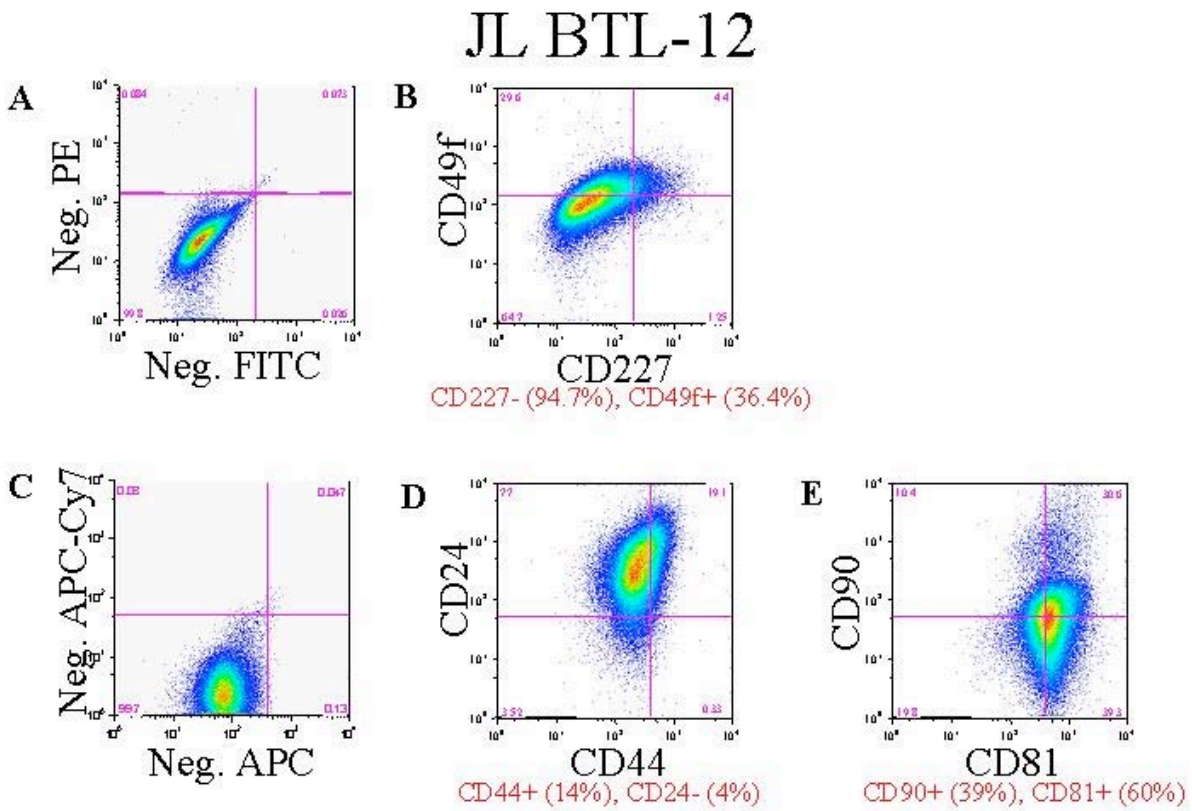


**Fig. 10.** Analysis by flow cytometry of the 12th Breast Tumor Line established in the Latimer Lab. Cells were stained with CD227 (Muc-1)-FITC, CD49f (alpha-6 integrin)-PE, CD44-APC and CD24/anti-mouse-bio/Streptavidin Cy7-APC or CD81 (TAPA-1)-APC and CD90-bio (Thy-1)/Streptavidin Cy7-APC). Dot plots represent the gated viable cells (PI- cells).

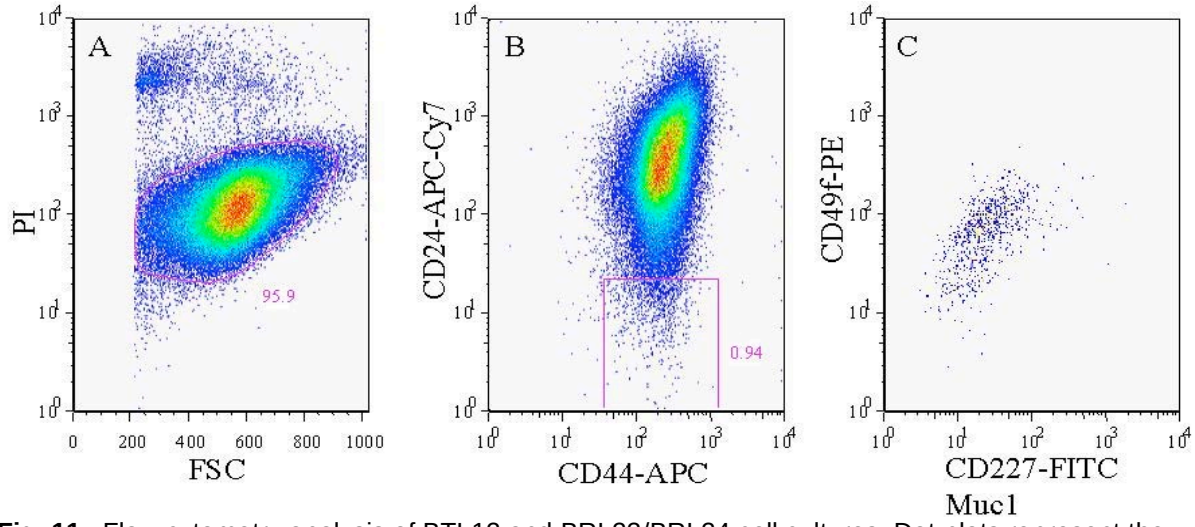


**Fig. 11.** Flow cytometry analysis of BTL12 and BRL23/BRL24 cell cultures. Dot-plots represent the gated viable cells (PI negative cells). Cells were stained with CD227 (Muc-1)-FITC, CD49f ($\alpha$-6 integrin)-PE, CD44-APC and CD24/anti-mouse-bio/Streptavidin Cy7-APC or CD81 (TAPA-1)-APC and CD90-bio (Thy-1)/Streptavidin Cy7-APC). 0.94% of JL BTL-12 population represent CD24 -, CD44+. These cells were gated out as shown and re-analyzed with CD49f and CD227. All but 4 were CD49f+ and CD227-. This means that only a subset of cells from BTL12 are CD44+CD24-, cell surface markers identified as part of the "tumorigenicity markers" for primary beast cancer cells.

*3. Transduce flow sorted stem cell population with a Green Fluorescent Protein (GFP) expressing viral construct and reintroduce these cells at the same time point of differentiation into primary culture system in order to determine the functionality of these cells in the differentiation of organotypic architecture. In effect, we will perform lineage tracing with the expression of GFP. We will document this lineage tracing by creating live cell digital microscopy movies under fluorescence optics.*

We have determined that we can actually clone the stem cell compartment of our normal cell lines. At the time we wrote the grant we did not assume that this would be possible (in fact we assumed that it would be impossible). Cloning efficiencies are shown for normal cell line, BRL 24 in Table 1, for a pre-invasive DCIS cell line and and for a tumor cell line BTL-12 . We have been able to clone several of our cell lines which is direct evidence of the presence of stem cells and we have seen bi-potent differentiation of 2 cell types (Fig. 12 ) from a single cells in our normal cell line JL BRL 24. This is the strongest evidence to date that stem cells are being maintained in our culture system.

We will perform the transduction in addition to what we have proven with cloning but it will take place in the time remaining on the grant. Several publications are being prepared now that specifically address the stem cell aspect of our work.

# Cloning Efficiency

| CELL LINE | CONDITIONS | CLONING EFFICIENCY |
|---|---|---|
| BRL24 | Plastic, Unconditioned Medium | 12.2% |
| BRL24 | Matrigel, Unconditioned Medium | 12.2% |
| DCIS3B | Plastic Conditioned Medium | 9.6% |
| DCIS3B | Matrigel, Conditioned Medium | 49.0% |
| DCIS3B | Plastic, Unconditioned Medium | 6.1% |
| DCIS3B | Matrigel, Unconditioned Medium | 4.1% |
| BTL12 | Plastic Conditioned Medium | 49.2% |
| BTL12 | Matrigel, Conditioned Medium | 49.2% |
| BTL12 | Plastic, Unconditioned Medium | 11.1% |
| BTL12 | Matrigel, Unconditioned Medium | 10.9% |

**Table 1**. Cloning Efficiency of 3 human cell lines established in Dr. Latimer's laboratory. BRL-24 is a non diseased Breast Reduction Cell Line, DCIS 3B is a Ductal Carcinoma In Situ Cell line which is unprecedented in the literature, BTL 12 is a stage III breast tumor cell line. Both tumor cell lines are chemotherapy naive. 4 different conditions were tested for 2 of these cell lines but cloning is achievable for all.


**Fig. 12.** Single cell pick up was physically performed for the BRL 24 cell line. Confirmation of a single cell in each well was confirmed microscopically. A few days later, the appearance of 2 cells, clearly a fibroblast and an epithelial cells were present in one of the wells of a 96 well plate that had previously contained a single cell. Although this needs to be repeated, it is strong evidence of the presence of multipotent stem cells in this non-diseased culture.

4. Write the data up for publication and presentation at AACR and the ERA of hope meetings. Some of this work was presented at the AACR 2006 meeting.
**See the Reportable outcomes below.**

## Key Research Accomplishments:

Evidence to support our hypothesis that stem cells are being maintained in our model system:

1.) De novo organotypic formation of ducts and lobules in vitro.

2.) Continuous growth of non-diseased cells while maintaining a normal karyotype (data not shown).

3.) Non-diseased cells maintain long telomeres after 20 passages (data not shown).

4.) Cells present in both non-diseased and tumor cell lines that are consistent with stem cells in terms of flow sorting markers.

5.) In vitro differentiation of 2 cell types (pluripotency) from a single cell in cloning experiments (Fig. 12 )

## Reportable Outcomes:

Papers:

Kelly, C.M. and **Latimer, J.J**. (2005) Unscheduled DNA synthesis: a functional assay for global genomic nucleotide excision repair. *Methods in Molecular Biology* **291**: 303-320.

Johnson, J.M., and **Latimer, J.J**. . (2005) Analysis of DNA repair using transfection-based host cell reactivation. *Methods in Molecular Biology* **291**: 321-335.

**Latimer**, **J**.**J**., Rubinstein, W.S., Johnson, J.M., Kanbour-Shakir, A., Vogel, V.G., and Grant, S.G. (2005) Haploinsufficiency for *BRCA1* is associated with normal levels of DNA nucleotide excision repair in breast tissue and blood lymphocytes. *BMC Medical Genetics* **6**(26) online.

Rubinstein, W.S., **Latimer, J.J.**, Sumkin, J., Huerbin, M.B.. Grant, S.G. and, Vogel, V.G., A study of 0.5 Tesla dedicated magnetic resonance imaging for the detection of breast cancer in young high-risk women. *BMC Women's Health* (in press).

Meeting Abstracts:

**Latimer, J.J**., Johnson, J.M., Kelly, C.M., Grant, S.G., Vogel, V.G., Brufsky, A.M., and Kelley, J. (2004) Human breast cancer tumors manifest both hereditary deficiency and somatic loss of DNA (nucleotide excision) repair. Presented at the 2004 meeting of the *Environmental Mutagen Society*, Pittsburgh, Pennsylvania. *Environmental and Molecular Mutagenesis* **44**: 211.

Grant, S.G., Wenger, S.L., Rubinstein, W.S**., Latimer, J.J**., Bigbee, W.L., and Auerbach, A.D. (2004) Elevated levels of somatic mutation in homozygotes and heterozygotes for inactivating mutations in the genes of the FA/BRCA DNA repair pathway. Presented at the 2004 meeting of the *American Society of Human Genetics*, Toronto, Canada. *American Journal of Human Genetics* **75**(**supplement**): 94.

**Latimer**, **J**.**J**., Johnson, J.M., Kelly, C.M., Mehta, S.B., Grant, S.G., Vogel, V.G., and Kelley, J. (2004) Loss of expression of CSB and XPA in both hereditary deficiency and somatic loss of DNA (nucleotide excision) repair in human breast cancer. Presented at

the American Society for Microbiology conference on *DNA Repair and Mutagenesis: From Molecular Structure to Biological Consequences*, Southampton, Bermuda.

Grant, S.G., Johnson, J.M. and **Latimer**, **J**.**J**. (2005) Genetic basis of DNA repair deficiency in sporadic breast cancer. Presented at *A Promise In Action—The Susan G. Komen Breast Cancer Foundation 2005 Mission Conference*, Washington, D.C.

Johnson, J.M., and **Latimer**, **J**.**J**. (2005) Molecular mechanism of nucleotide excision repair deficiency in novel breast tumor cell lines. Presented at *A Promise In Action—The Susan G. Komen Breast Cancer Foundation 2005 Mission Conference*, Washington, D.C.

**Latimer**, **J**.**J**., Johnson, J.M., Kelly, C.M., Beaudry-Rodgers, K., Vogel, V.G., Kelley, J., Johnson, R., Amortegui, A., Mock, L. and Grant, S.G. (2005) Genetic analysis of DNA nucleotide excision repair deficiency in novel non-tumor adjacent and tumor cell lines suggests a new paradigm of breast cancer etiology. Presented at the 2005 *Department of Defense Research Program Era of Hope Meeting*, Philadelphia, Pennsylvania.

Grant, S.G., Kelley, J.L. III, Vogel, V.G., Brufsky, A.M., Bigbee, W.L., and **Latimer, J.J.** (2005) Variability in bone marrow mutational response in breast cancer patients treated with genotoxic chemotherapy. Presented at the joint meeting of the *9th International Conference on Environmental Mutagens* and the 2005 annual meeting of the *Environmental Mutagen Society*. *Mutation Research* **577**(**Supplement 1**): e165.

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**Latimer, J.J.**, Benson, A., Lagasse, E., Johnson, J.M., Kelley, J.L. III, Davis, D.L., Beer Stolz, D. (2006) Identification of stem cells in a novel human mammary epithelial tissue engineering system that reproducibly demonstrates ductal organotypic architecture. Presented at the American Association for Cancer Research Meeting, Washington, D.C. *Proceedings of the American Association for Cancer Research* **47**: 320.

## Conclusions:

We have collected compelling evidence to show that our laboratory is able to maintain stem cells in culture from the non-diseased human breast. With these cells we will be able to test a widely held hypothesis that breast cancer arises from stem cells in the breast. We have also been able to achieve the first stages of in vitro transformation in a non-diseased breast reduction cell line and hope to have fully transformed cells soon (from Benzo [a] pyrene dialoepoxide (BPDE) exposure at 5 uM (data not shown). We can then use isolated stem cells for a similar experiment in the near future.

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## Appendices

## Haploinsufficiency for BRCA1 is associated with normal levels of DNA nucleotide excision repair in breast tissue and blood lymphocytes

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# Haploinsufficiency for *BRCA1* is associated with normal levels of DNA nucleotide excision repair in breast tissue and blood lymphocytes

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# Abstract

### Background:

Screening mammography has had a positive impact on breast cancer mortality but cannot detect all breast tumors. In a small study, we confirmed that low power magnetic resonance imaging (MRI) could identify mammographically undetectable tumors by applying it to a high risk population. Tumors detected by this new technology could have unique etiologies and/or presentations, and may represent an increasing proportion of clinical practice as new screening methods are validated and applied.

### Case presentation:

We describe a breast cancer patient with a mammographically undetectable stage I tumor identified in our MRI screening study. She was originally considered to be at high risk due to the familial occurrence of breast and other types of cancer, and after diagnosis was confirmed as a carrier of a Q1200X mutation in the *BRCA1* gene. In vitro analysis of her normal breast tissue showed no differences in growth rate or differentiation potential from disease-free controls. Analysis of blood lymphocyte and breast epithelial cell samples with the unscheduled DNA synthesis assay revealed no deficiency in nucleotide excision repair (NER).

### Conclusions

As new breast cancer screening methods become available and cost effective, patients such as this one will constitute an increasing proportion of the incident population, so it is important to determine whether they differ from current patients in any clinically important ways. Despite her genetic status, and her mammographically dense breast

tissue, we did not find increased cell proliferation or deficient differentiation potential in her breast epithelial cells, which might have contributed to her cancer susceptibility. Although NER deficiency has been demonstrated repeatedly in blood samples from sporadic breast cancer patients, analysis of blood lymphocytes and breast epithelial cells for this patient proves definitively that heterozygosity for inactivation of *BRCA1* does not intrinsically confer this type of genetic instability. These data suggest that the mechanism of genomic instability driving the carcinogenic process may be fundamentally different in hereditary and sporadic breast cancer, resulting in different genotoxic susceptibilities, oncogene mutations, and a different molecular pathogenesis.

## Background

A reduction in breast cancer mortality has been observed in recent years that has been partially attributed to the widespread adoption of screening mammography [1]. Traditional screening mammography, however, fails to detect 15% of incident cancers [2]. New, complementary imaging techniques are therefore under development that may increase the accuracy of primary screening. We performed a small study to validate the use of low power magnetic resonance imaging (MRI) to prospectively detect breast alterations and malignancy and to determine the feasibility of applying this technique to a high-risk population [3]. We present here a subject from that study whose early stage tumor was not detectable by mammography.

This patient was enrolled in the screening study due to her family history of breast and other neoplasias. After tumor diagnosis, she was determined to be heterozygous for a putative inactivating mutation in the *BRCA1* gene. In addition, she had dense breast tissue, an impediment to mammography that is in itself a risk factor for breast

cancer [4]. Breast development and lactational differentiation also appear to individually modify breast cancer risk, with early term pregnancy conferring a persistent protective effect [5]. Exposure to ionizing radiation, while a lifetime risk factor for breast cancer, appears to be more dangerous when it occurs during alveolar differentiation of the breast at adolescence [6]. Using a novel tissue engineering system [7], we therefore examined the growth and differentiation of normal breast epithelial samples from this patient via live-cell imaging.

The *BRCA1* hereditary breast cancer gene has been shown to be involved in DNA double strand break repair [8,9]. DNA repair defects have also been identified in the peripheral blood cells of sporadic breast cancer patients [10-13], but, in this case, it seems to involve a different pathway of DNA repair, nucleotide excision repair (NER) [14-16]. We have extended this observation of NER deficiency to the tumor itself, as well as the adjoining non-diseased normal breast tissue [17]. It has recently been shown that *BRCA1* expression can enhance NER activity, although this analysis was not performed in breast cells [18,19]. We therefore applied the functional UDS assay for NER capacity to multiple samples of normal tissue from this patient, to determine whether haploinsufficiency for *BRCA1* was a mechanism of NER deficiency.

# Case presentation

We describe a breast cancer patient whose tumor was detected by MRI. She was enrolled into a pilot screening study of low power MRI due to her familial risk. She had mammographically dense breasts and her tumor was undetectable mammographically.

### Patient description

The patient was a 35.7 year old woman who presented with a very strong family history of breast cancer as depicted in Figure 1, and negative physical and mammographic examination. She had extremely dense breast tissue bilaterally by mammography as well as fibrocystic breast tissue by physical examination. She had no previous personal history of breast biopsy or abnormal mammograms.

### Risk profile

The 5 year breast cancer risk for this patient as calculated by the BRCAPRO model was 5.7%, and her probability of being a *BRCA1* or *BRCA2* carrier was 0.47. The Gail model risk assessment was calculated using the following information: Race-Caucasian; Age-35; Age at first menses-12; Age at first live birth-nulliparous; Number of first-degree relatives with breast cancer-2; Number of previous breast biopsies-0. The calculated 5 year Gail risk was 1.0% and her lifetime risk was 31.3%.

### Genetic testing

Following genetic counseling, the patient elected to undergo DNA sequencing of the *BRCA1* and *BRCA2* genes, which revealed a Q1200X truncation mutation in one of her *BRCA1* alleles. The C to T mutation at codon 1200 in exon 11 results in the change of the amino acid glutamine to a stop codon with resulting protein truncation

and loss of function. Exon 11 is the largest exon in *BRCA1* and has the highest frequency of reported mutations. The Q1200X mutation has been independently observed several times [20].

### Imaging

The bilateral screening mammogram was compared to previous films from another hospital. The breast tissue was described as heterogeneously dense, thus lowering the sensitivity. There were no masses, significant calcifications or other findings and the mammogram was interpreted as negative bilaterally. A one-year follow-up was recommended.

The patient was then MRI scanned as previously described [3], with pre- and post-gadolinium enhancement images evaluating both breasts simultaneously in the axial plane. In the upper-outer left breast there was a small (approximately 1 cm), round, well-demarcated enhancing lesion. This lesion was seen on both the initial delay after contrast injection and the delayed contrast enhanced subtraction images. The lesion appeared to accumulate contrast to a greater extent on the delayed subtract ion images with an additional lesion adjacent to the first. In the medial aspect of the mid right breast, there were several small punctate areas of enhancement on both the immediate and delayed subtraction views. Also in the right breast just above the nipple level medial and close to the chest wall an additional enhancing lesion was seen. This lesion was approximately 1.5 cm, round, and well-demarcated which continued to accumulate contrast on the delayed subtraction images. This lesion appeared to have a small non-enhancing septation.

### Core biopsies

Under ultrasound, the lesion of concern in the left breast was identified and biopsied, as well as one lesion in the right breast (Figure 2). The core biopsy of the left breast revealed infiltrating ductal carcinoma in 2 of 5 core fragments; high nuclear grade, with no lymphatic invasion seen. The core biopsy of the right breast demonstrated benign pathology, specifically, fibrosis with focal ductal epithelial hyperplasia.

### Final pathology, treatment plan and outcome

Although a surgical candidate for lumpectomy and radiation, the patient chose to undergo left modified radical mastectomy with left axillary lymph node dissection and contralateral prophylactic total mastectomy because of her genetic risk status. The pathology in the left breast was consistent with the imaging and core biopsy in size and description. Tumor size was 8 mm in greatest dimension, nuclear grade III, ER/PR and Her2/neu negative, and the nodal status (0/4) was negative (stage TIaN0M0). The patient underwent 4 cycles of chemotherapy and has been reportedly healthy since. Because of the positive *BRCA1* mutation results, she subsequently underwent prophylactic bilateral salpingo-oophorectomy.

### Live-cell analysis of tissue explant cultures

A number of life history factors have been associated with breast cancer incidence that are widely interpreted as representing lifetime exposure of the breast tissue to estrogen-induced mitogenesis [21]. An alternative interpretation, based on epithelial cell differentiation, suggests that lactational differentiation, such as occurs during term pregnancy, confers resistance to carcinogenesis [22,23]. We have developed a novel breast tissue engineering system wherein many aspects of organotypic

differentiation are reiterated in vitro [24]. In this system, breast epithelial cells initially retain cell-to-cell contact while they proliferate, then undergo an architectural reorganization, first to form three-dimensional mammospheres, and later vast networks of branching ductal and lobular structures. Tumor and some pre-neoplastic samples fail to form such architecture. Normal tissue from this patient, who is both a *BRCA1* mutation carrier and has dense breasts, was evaluated to determine whether either of these factors affected de novo differentiation in this system. Four discrete pieces of fresh tissue were provided for live-cell analysis from each of the patient's ipsilateral and contralateral breasts. In the case of the ipsilateral breast, this tissue was provided at increasing distance from the tumor margin in 1 cm increments. All of these normal samples attached and grew in our culture system and were examined for cell-to-cell interactions and morphology over a period of one month. In the context of breast reduction epithelium (BRE) tissue samples from 22 patients with no breast disease, these patient samples manifested typical mixtures of fibroblastic and epithelial cells. After several days in culture without passaging, the epithelial cells began to self-organize, initially forming three-dimensional mammospheres (Figure 3A), and, after 2 weeks in culture, more complex pre-ductal linear columns of epithelial cells (Figure 3B). The tissue explants from both breasts showed similar patterns of behavior (Figure 3). Tissue cultured from a contemporaneous disease-free control and the contralateral breast of a sporadic breast cancer patient showed similar morphology and architecture (data not shown).

### Cell growth kinetics

It has been suggested that the association between breast density and risk of breast cancer is due to increased cell proliferation [25]. One measure of cell growth and viability is the S-phase index (SPI) or the percentage of cells incorporating

radiolabeled thymidine over a specific incubation period (in our case, 2 hours). In a previous study with 22 normal BRE samples we observed a wide range of proliferation rates, with SPI ranging from a low of 0.2% to a high of 46.0% (mean of 18.3 ± 2.6%) [26]. The contemporaneous control sample from a disease-free breast reduction patient had an SPI of 30.9%, at the higher end of this normal range. The ipsilateral and contralateral tissue samples from the hereditary breast cancer patient exhibited SPI of 26.6% and 26.2%, respectively, placing them at slightly over the 70$^{th}$ percentile for growth rate. The contralateral sample from the sporadic breast cancer patient had an SPI of 17.0%, placing it slightly under the 50$^{th}$ percentile. Thus, all of these breast cancer patient samples appeared to grow well in our system, with SPI well within the range of our normal samples. The similarity of the SPI values from the two samples from the *BRCA1* mutation carrier does not appear to be accidental; the chances of selecting two samples from the normal population with values as close or closer is very small ($P = 0.026$).

### Functional analysis of NER capacity

Peripheral blood lymphocytes and normal breast epithelial tissue from the hereditary cancer patient were then cultured for performance of the functional UDS assay, which requires living cells for radiolabel incorporation during DNA repair synthesis following UV exposure. This assay is diagnostic for the inherited cancer-prone disease xeroderma pigmentosum, where it is usually performed in lymphocytes or skin fibroblasts. Our novel HME tissue engineering system allows us to apply the assay to breast epithelial cells, and we have previously demonstrated tissue-specificity in the NER capacity of these cells in normal samples from patients undergoing breast reduction mammoplasty [26]. Patient data is therefore expressed relative to the average of our breast reduction controls.

Analysis of cultured blood lymphocytes from the patient established that they had normal NER capacity (99.6% of the average of our 33 normal samples) (Figure 4). This is well above the cut-off established in our sporadic breast cancer population, < 70% average normal activity, which when applied to our cases and controls yielded a significant odds ratio of 37.4 [27]. A trend towards age dependence had been noted in the analysis of the UDS data of the normal controls ($P = 0.059$) [26]; addition of the patient sample supports this trend, but it still fails to reach significance ($P = 0.056$).

The functional NER assay was then applied to the contemporaneous disease-free breast reduction control sample, one sample each from the ipsilateral and contralateral breasts of the patient, and to a sample from the contralateral breast of an apparently sporadic breast cancer patient. The NER of the BRE non-diseased control was 1.82 times the average of our normal data set for this tissue and within the range of normal. The NER capacity of the ipsilateral breast epithelial sample was 1.05 times the average of our population of BRE controls, clearly exhibiting no overt DNA repair deficiency (Figure 5). The contralateral sample was very similar, with an NER capacity of 1.17 times BRE normal. Although the NER values of these two samples from the same patient are similar, they are not close enough to distinguish themselves as coming from the same individual ($P = 0.16$). The NER capacity of the contralateral sample from the sporadic breast cancer patient was 1.62 times the average of the BRE controls, also in the normal range.

Our earlier analysis of NER in our normal population revealed no effects of age or cell proliferation (as represented by the S-phase index). All of these additional patient samples are consistent with those results.

## Discussion

At least two types of breast tumors are not accurately detected by traditional screening mammography: "interval" tumors that arise quickly between screenings and tumors whose density is not sufficient to distinguish them from the surrounding normal tissue. The latter situation is more likely to occur in women with dense normal breast tissue, which, in turn, is more typical of younger women. Thus, mammographically undetectable tumors may have a number of characteristics, such as fast growth, early onset and/or occurrence in dense breasts that might distinguish them from mammographically detectable tumors in terms of molecular etiology and clinical parameters of prognosis and response. The present patient had an early onset breast tumor, but had both hereditary susceptibility due to her *BRCA1* mutation and dense breasts, so her presentation is not unusual in this context. It is possible that breast tumors detected by complementary screening methods in the future will demonstrate unique clinical and molecular features, when it becomes feasible to perform such screening in the general population.

Since the *BRCA1* gene product is known to play a role in DNA double strand break repair [8,9], it has been suggested that decreased repair capacity is the basis of the breast cancer predisposition observed in mutation carriers [28-31]. Such a cellular phenotype has been difficult to demonstrate, however [32-35]. An alternate possibility is that the mutation affects the growth or differentiation of breast epithelial cells in a manner consistent with cancer susceptibility. It has been suggested that dense breast tissue is indicative of generalized hyperproliferation that might promote oncogenesis [25]. Our findings show that all 8 samples, derived from both the involved and the uninvolved breasts of a hereditary breast cancer patient develop normal epithelial architecture in vitro, implying that the epithelial/stromal (paracrine)

interactions necessary for the development of this complex architecture are intact and normal in *BRCA1* heterozygotes despite their greater risk of breast cancer. The SPI results also indicate that this non-diseased epithelial tissue falls into the typical range of normal for BRE control cultures and is demonstrating typical growth in our HMEC system.

NER deficiency is most often associated with XP, sensitivity to UV-induced DNA damage and skin cancer [13-16]. The NER deficiency of XP patients is manifested in other tissues, however, as shown by their high spontaneous frequency of mutation in blood lymphocytes [36] and the occurrence of other types of tumors [37]. The observation that sporadic breast cancer patients have low levels of NER in peripheral lymphocytes suggests that sporadic breast cancer is associated with constitutively low levels of NER [14-16]. Our results from a single patient demonstrate, however, that while overexpression of *BRCA1* may enhance NER [18], haploinsufficiency for this gene does not necessarily result in detectable NER deficiency. Since it is clear that genomic instability is a necessary prerequisite for the completion of the complex multi-step carcinogenic pathway(s) involved in breast cancer, a fundamental difference in the mechanisms of genomic instability arising in hereditary and sporadic breast tumors would be likely to translate into fundamentally different patterns of molecular pathogenesis that could impact on clinical management.

## Conclusions

This patient and her tumor represent the vanguard of a new population of early stage breast cancer patients that will be increasingly diagnosed as new screening technologies complementary to mammography are validated and become practicable. We have shown that low power MRI can detect a stage I tumor in dense breast tissue;

the same technology can also impact upon interval tumors by staggering the procedure with mammography rather than applying them coincidently. Although we did not observe obvious differences in the growth rate or differentiation potential of the dense breast tissue from this patient, we cannot rule out the possibility that some or all of the tumors detectable only by complementary screening procedures will differ from the present clinical experience in important ways. Our live-cell analysis takes a step toward defining cellular characteristics that may be useful for cancer risk assessment, but we are only beginning to investigate the possibilities of the system. It may be that different growth conditions, or induction with genotoxic or estrogenic agents, will allow for the greater differentiation of breast tissue and tumor behaviours. This technique also allows for the application of functional assays to patient samples, as exemplified in this report by the UDS assay for NER capacity. Those UDS results, although from a single patient, demonstrate definitively that the constitutively low NER capacities reported in several sporadic breast populations do not arise as a pleiomorphic effect of *BRCA1* haploinsufficency. Thus, the basis of genetic instability, a fundamental element in breast carcinogenesis, may differ between sporadic and hereditary breast tumors, resulting in different susceptibilities to inducing agents, mutations in different sets of oncogenes and tumor suppressor genes, and, ultimately, tumors of different molecular etiology that express different clinically relevant phenotypes.

# Methods

### Patients and controls

The patient was a 35.7 year old woman with strong family history of breast cancer recruited into a clinical trial of MRI screening for young woman at high risk for breast

cancer with dense breast tissue [3]. Gadolinium enhancement images revealed a small 1 cm lesion in the upper-outer quadrant of the left breast, identified pathologically as an infiltrating ductal carcinoma. The patient underwent a modified radical mastectomy of the left breast and chose to also undergo a contralateral prophylactic total mastectomy. Blood and tissue were obtained for analysis with consent under Magee-Womens Hospital (of the University of Pittsburgh Medical Center) IRB # MWH-94-108.

Data from this hereditary breast cancer patient were compared to that from two additional patients as well as previously published controls. The first new control patient was a 20 year old women undergoing breast reduction mammoplasty. The second contemporaneous control patient was a 36 year old woman undergoing cosmetic surgery on her contralateral breast two years after successful lumpectomy to remove an apparently sporadic stage IIA breast tumor (2.5 cm, negative for estrogen and progesterone receptors, 13 lymph nodes negative). She had undergone standard radiotherapy and chemotherapy with adriamycin and cyclophosphamide. Histopathological analysis confirmed that the breast tissue from both of these control patients was free of cancer and within the acceptable histological range of normal.

### Patient tissue culture and analysis

Fresh tissues from the patient were obtained within 5 hours of surgery. After pathological evaluation, excess tissue not needed for diagnosis was placed into DMEM containing 10% fetal calf serum and 3x antibiotic antimycotic (Sigma, St. Louis, MO) at 4ºC. This tissue was then processed as described in Latimer *et al*. [26] and placed into culture on a diluted form of matrigel (1:1 with DMEM) in the novel MWRI medium [7].

Eight samples of the principal patient's tissue were obtained for culture after bilateral mastectomy surgery. We were not able to obtain a sample of her tumor, because it was utilized entirely for clinical diagnosis. We were able to obtain 4 pieces of histologically normal non-tumor adjacent tissue at increasing 1 cm intervals from the tumor margin from her left (ipsilateral) breast. In addition, we obtained 4 similar pieces of fresh tissue from her contralateral breast. All were placed into primary explant (HMEC) culture.

For analysis of cell growth and in vitro differentiation, explants were cultured and imaged every second day using a digital Hamamatsu Orca video camera for 30-60 days. Images were analyzed on a Macintosh G4 computer using QED imaging software (Media Cybernetics, Inc., Silver Spring, MD).

### Control tissue cultures

Breast reduction mammoplasty tissues were obtained from patients ages 20-70 at Magee-Womens Hospital under the above IRB. A neighboring piece of mammoplasty tissue (from the same 0.25 $cm^2$ sample) to that placed into primary culture was fixed and processed in paraffin. These sections were examined by a pathologist to verify the histological features and normality of the tissue. Breast tissue was processed as previously described [26]. Tissue was rinsed three times in PBS containing antibiotics, physically disaggregated and placed into MWRIα medium [7] on a thin coat of matrigel. Peripheral blood lymphocytes (PBLs) were obtained with consent from normal healthy control subjects ages 20-50 working at Magee-Womens Hospital or students at the University of Pittsburgh. Foreskin fibroblast (FF) tissue was obtained as discarded tissue from newborn infants after circumcision and utilized between passages 7 and 10. These control populations have

been previously described in greater detail [26,38]. Breast tissue samples from the two new control patients were processed in the same manner.

### Analysis of S-phase indices

Primary cultures of mammary tissue, established 10-14 days, were labeled with $^{3}$H-thymidine for a period of 2 hours followed by a chase with cold thymidine for 2 hours and then processed for autoradiography. After a 10-12 day exposure, slides were processed and analyzed by 2 independent, blinded scorers who evaluated the tissue samples for the percentage of cells in S phase (characterized by complete coverage of the nucleus with silver grains).

### Unscheduled DNA synthesis

NER was measured by autoradiography of unscheduled DNA synthesis after UV damage (UDS) [39,40]. After a total of 10-14 days in culture, without passaging, cultures were irradiated with UV light at 254 nm at a mean fluence of 1.2 Joules/$m^2$ for 12 seconds in the absence of culture medium, for a total dose of 14 J/$m^2$. Each sample was represented by at least two chamber slides. One chamber of each 2-chamber slide was shielded from the UV dose to be used as an unirradiated control sample. Primary cultures had not reached confluence and were still actively growing at the time the UDS assay was performed. Control FF were plated subconfluently 2 days before the UDS assay to insure that they also were not in a quiescent state brought on by confluence. After UV exposure, all cultures were incubated in medium supplemented with 10 μCi ml [$^{3}$H] methyl-thymidine (~80 Ci mmol$^{-1}$) (PerkinElmer Life Sciences, Boston, MA) for 2 hours at 37ºC. Labeling medium was then replaced with unlabeled chasing medium containing $10^{-3}$ M non-radioactive thymidine (Sigma) and incubated for a further 2 hours to clear radioactive label from the intracellular

nucleotide pools. After incubation in the post-labeling medium, cells were fixed in 1X SSC, 33% acetic acid in ethanol, followed by 70% ethanol and finally rinsed in 4% perchloric acid overnight at 4ºC. All slides were dried and subsequently dipped in photographic emulsion (Kodak type NTB2) and exposed for 10 to 14 days in complete darkness at 4ºC.

The length of exposure of emulsion was determined in each experiment by preparing FF "tester" slides. After 10-12 days these tester slides were developed and grain counting was performed. If the nuclei over the foreskin fibroblasts averaged 50 or more grains per nucleus, then the rest of the experimental slides were developed. If the grain count was below this level, the remaining slides were left to expose 1-3 days longer before being developed.

### Grain counting

After photographic development of emulsion, all slides were stained with Giemsa, then examined at a total magnification of 1000X on a Zeiss Axioskop under oil emersion for grains located immediately over the nuclei of non-S phase cells [40]. Local background grain counts were evaluated in each microscopic field, over an area the same size as a representative nucleus, and this total was subtracted from the grain count of each nucleus in that field. The average number of grains per nucleus was quantified for each side of the chamber slide, both unirradiated and irradiated. The final NER value for each slide was calculated by subtracting the unirradiated mean grains per nucleus from the irradiated mean grains per nucleus, after the initial subtraction of local background in each field. NER was initially expressed as a percentage of the activity of concurrently analyzed FF. Four FF slides were scored per experiment, by an average of three counters. 200 nuclei were counted per slide, for a total of 800, with an average of 61.6 grains/nucleus. Six slides were evaluated

for the patient's PBL sample, two by each of three counters. An average of 195 nuclei were scored per slide (for a total of almost 1200), with an average of 7.5 grains/nucleus. Four slides were counted for the contemporaneous breast reduction control, two each by two counters. There were an average of 200 nuclei per slide and 14.1 grains/nucleus. Six slides were scored from the patient's ipsilateral breast tissue sample, two by each of three independent counters, and five slides were counted from the contralateral sample, again by three independent counters. An average of just over 100 nuclei were evaluated per slide for each sample, for a total of almost 600 nuclei for the ipsilateral sample and over 500 for the contralateral sample. As the NER capacities indicate, these samples had very similar counts; about 35 grains/nucleus for the ipsilateral sample and 28 grains/nucleus for the contralateral sample. Finally, four slides were counted from the contralateral sample of a sporadic breast cancer patient, by three counters. There were an average of 200 nuclei per slide and 29.4 grains per nucleus.

### Statistical analysis

To ensure accuracy and guard against transcription errors, raw grain counts from the UDS assay were processed independently in duplicate, once using StatView (version 5.0.1, SAS Institute, Inc., Cary, NC), and once using the Data Analysis Toolpack of the Excel 2001 spreadsheet program (Microsoft Corp., Redmond, WA). The final count from slides of the same cell type within the same experiment and developed the same day were averaged together and expressed as a percentage of concurrently analyzed FF. These results were then normalized by comparison to the average for the tissue type control population [56].

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

JJL conceived of the study, executed it, and drafted the manuscript. WSR recruited and consented the patient, provided clinical samples and information. JMJ evaluated the UDS assay and analyzed the data. AKS performed the histopathological analysis of the tissue. VGV participated in the study design and data interpretation. SGG participated in the design and coordination of the study and helped to draft the manuscript.

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## Figures

### Figure 1 - Pedigree of the patient (indicated by arrow)

She, one maternal aunt and one maternal cousin had breast cancer diagnosed at 36, 44 and 41 years old, respectively, as indicated by the half-filled symbols, and her aunt died of the disease. Her cousin underwent lumpectomy followed by chemotherapy, radiotherapy and is presently on tamoxifen. Her mother had breast cancer in both breasts, diagnosed at ages 41 and 42, as indicated by the completely filled symbol. She underwent bilateral mastectomy and hysterectomy followed by chemotherapy and radiotherapy and died of the disease at age 44. A second maternal aunt was diagnosed with colon cancer at age 52 (light half-filled symbol) and breast cancer at age 55 (dark half-filled symbol). Based on this pattern of familial cancer the patient was considered to be at high risk of developing breast cancer and was entered into the low power MRI screening validation and feasibility study. Following her diagnosis, she was confirmed as carrying a Q1200X mutation in the *BRCA1* gene.

### Figure 2 - Ultrasound of the MRI-detected lesion

Following MRI, the patient was scheduled for ultrasound to identify the questionable lesions seen on MRI for possible core biopsy. Under ultrasound the lesion of concern was identified and biopsied at the 1:00 location in the left breast. Additionally, one lesion seen by MRI in the right breast at the 4:00 location was identified and biopsied.

**Figure 3 - Micrographs of the non-diseased primary human mammary epithelial cultures (HMEC) from the *BRCA1* mutation carrier**

A) Contralateral breast - A cluster of epithelial cells called a mammosphere is shown on the left center of the image sitting on a field of fibroblasts. B) Ipsilateral breast - The original fresh tissue block from which this culture was derived was located 4 cm from the infiltrating ductal carcinoma. The structure shown is a cluster of rounded epithelial cells manifesting a column configuration called "pre-ductal linearization". Both images were captured under Differential Interference Contrast (DIC) optics on a Zeiss Axiovert 100 microscope at a total of 140x magnification.

**Figure 4 - Comparison of the NER capacity of a PBL sample from our *BRCA1* mutation carrier patient with those of a population of disease-free controls**

The dark horizontal line indicates the average for the normal population, while the dotted lines indicate upper limits for residual NER activity in patients with the hereditary NER deficiency disease XP (0.50) and the cut-off established in our breast tissue study that identified tumors with high sensitivity and specificity (0.70).

**Figure 5 - Comparison of the NER capacities of two samples of normal breast epithelium from our *BRCA1* mutation carrier patient with those of a population of disease-free controls who underwent breat reduction mammoplasty**

The dark horizontal line indicates the average for the normal population of breast reduction epithelium (BRE), while the dotted lines indicate upper limits for residual NER activity in patients with the hereditary NER deficiency disease XP (0.50) and the cut-off established in our breast tissue study that identified tumors with high sensitivity and specificity (0.70). The patient sample on the left was derived from the ipsilateral (left) breast, while the sample on the right was from the contralateral (right) breast.

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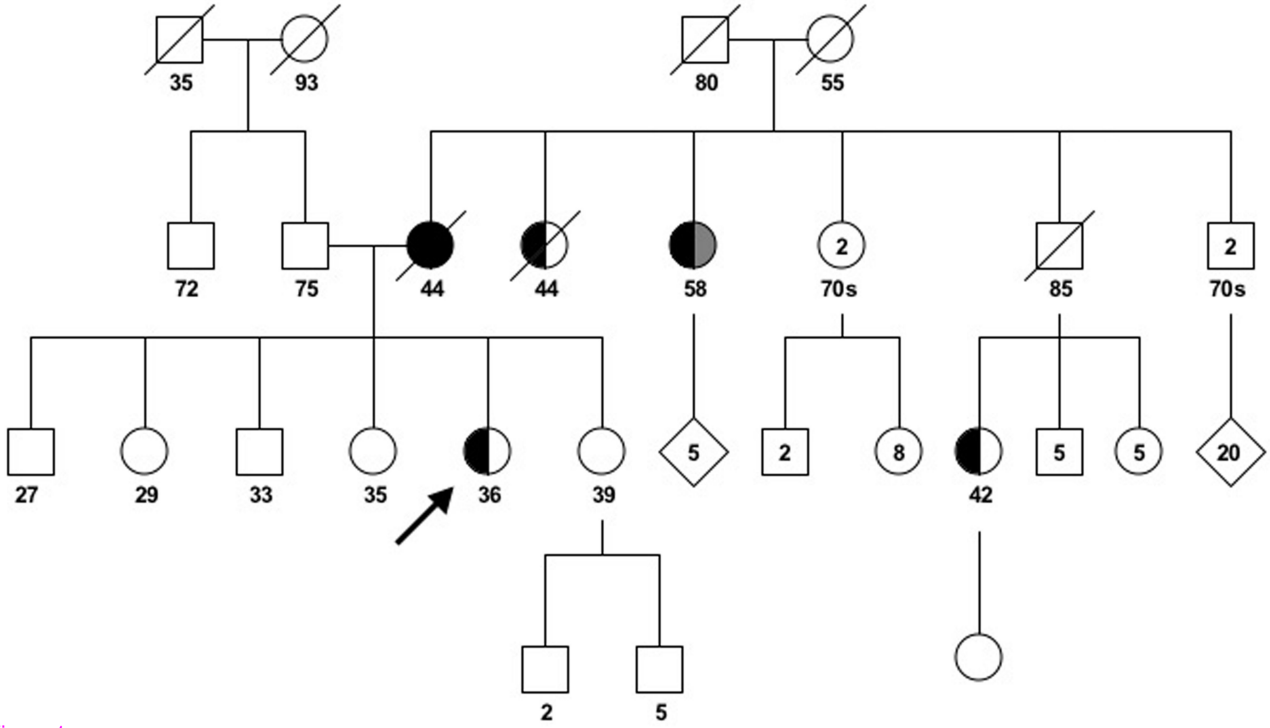
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Figure 1

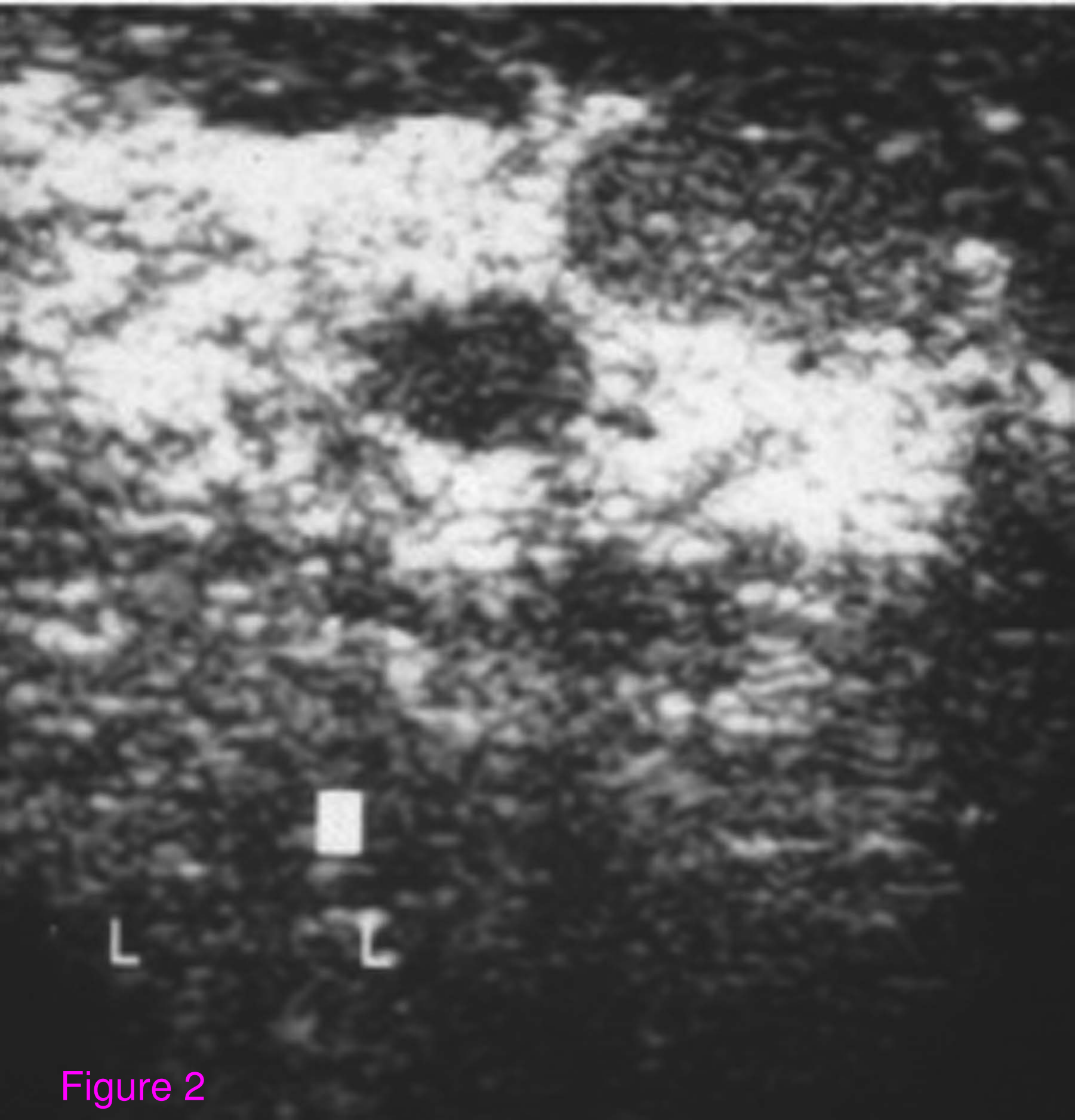


Figure 2

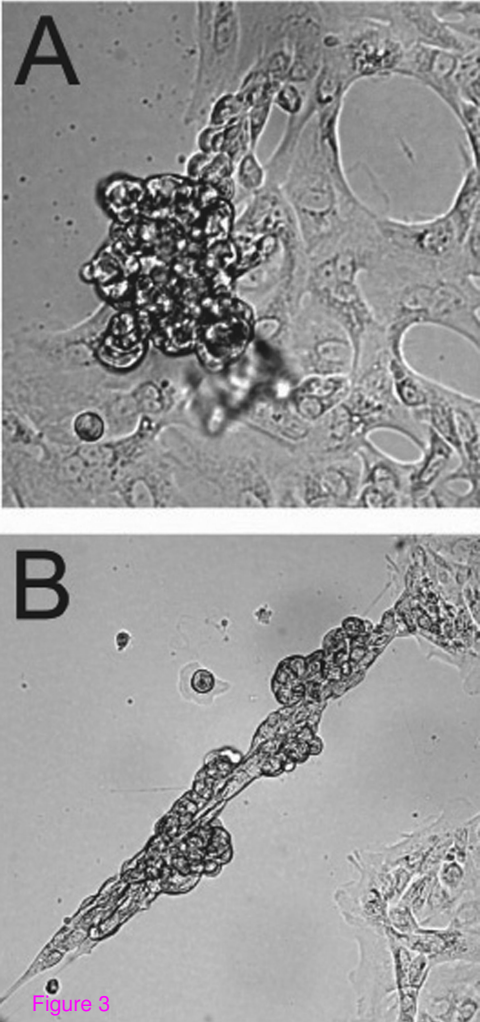


Figure 3

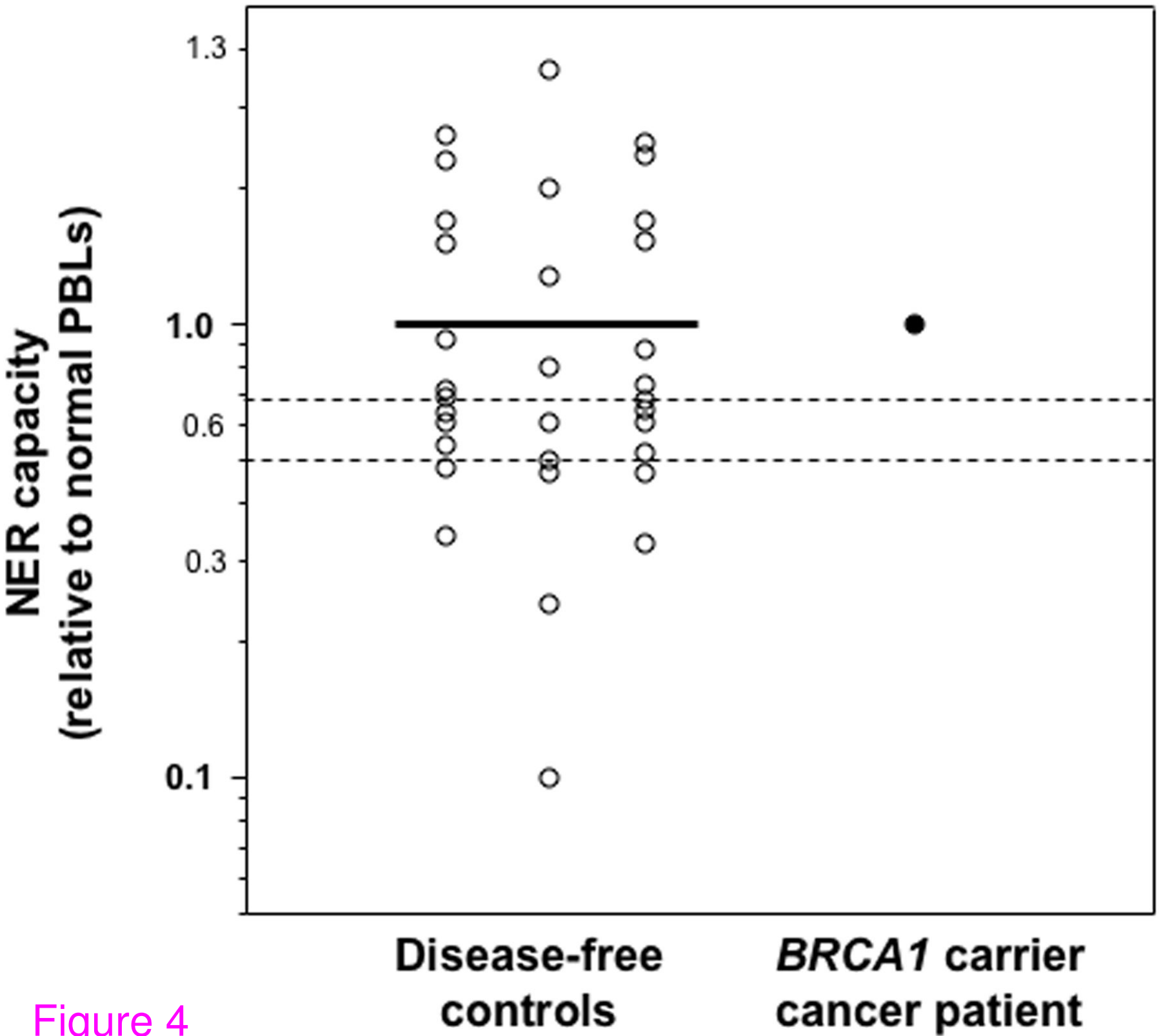
NER capacity
(relative to normal PBLs)
1.3
1.0
0.6
0.3
0.1
Disease-free
controls
BRCA1 carrier
cancer patient

Figure 4

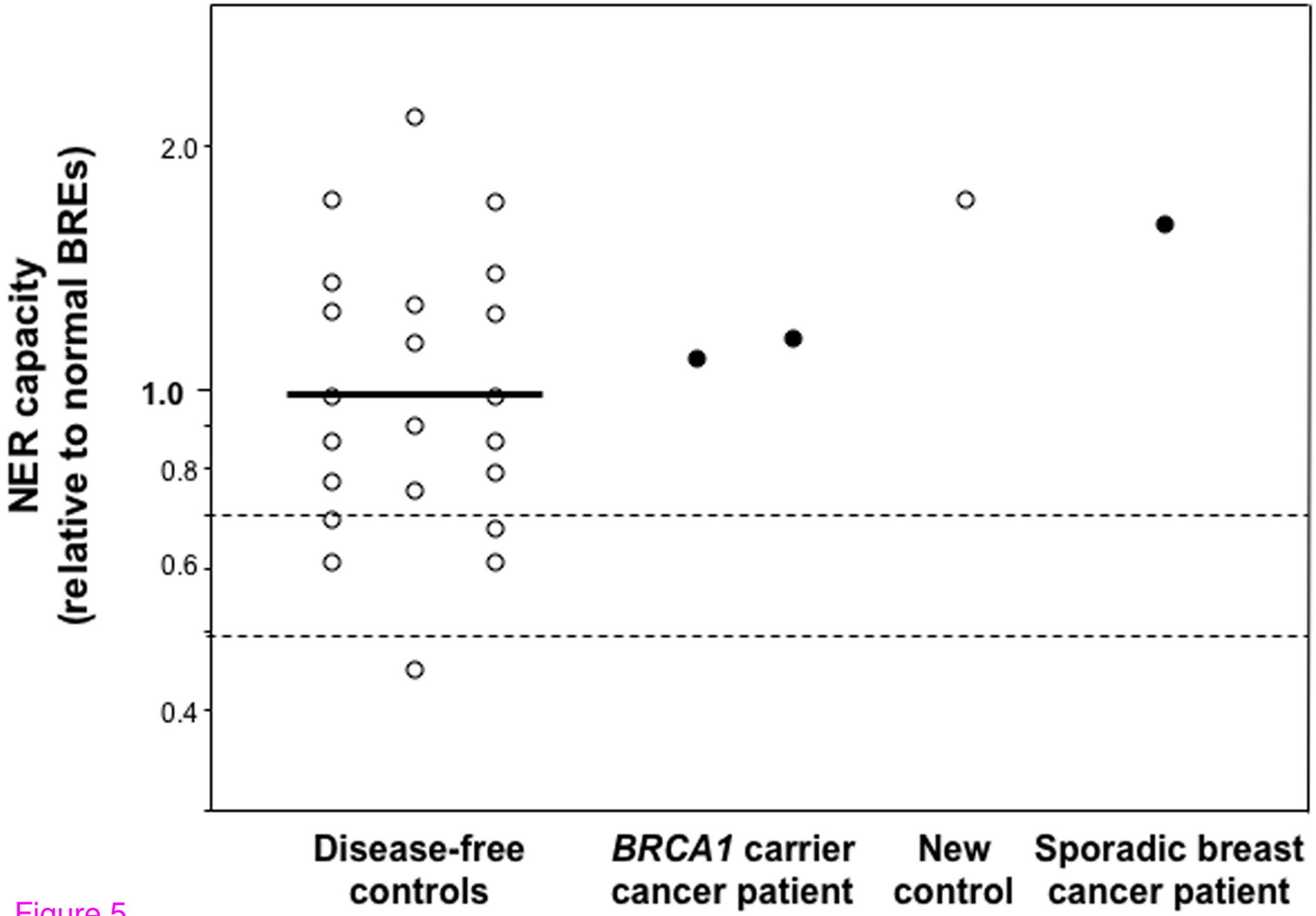
NER capacity
(relative to normal BREs)
2.0
1.0
0.8
0.6
0.4
Disease-free controls
BRCA1 carrier cancer patient
New control
Sporadic breast cancer patient

Figure 5