**In Vitro** Analysis of Breast Cancer Cell Line Tumourspheres and Primary Human Breast Epithelia Mammospheres Demonstrates Inter- and Intrasphere Heterogeneity

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**Abstract**

Mammosphere and breast tumoursphere culture have gained popularity as *in vitro* assays for propagating and analysing normal and cancer stem cells. Whether the spheres derived from different sources or parent cultures themselves are indeed single entities enriched in stem/progenitor cells compared to other culture formats has not been fully determined. We surveyed sphere-forming capacity across 26 breast cell lines, immunophenotyped spheres from six luminal- and basal-like lines by immunohistochemistry and flow cytometry and compared clonogenicity between sphere, adherent and matrigel culture formats using *in vitro* functional assays. Analyses revealed morphological and molecular intra- and intersphere heterogeneity, consistent with adherent parental cell line phenotypes. Flow cytometry showed sphere culture does not universally enrich for markers previously associated with stem cell phenotypes, although we found some cell-line specific changes between sphere and adherent formats. Sphere-forming efficiency was significantly lower than adherent or matrigel clonogenicity and constant over serial passage. Surprisingly, self-renewal capacity of sphere-derived cells was similar/lower than other culture formats. We observed significant correlation between long-term-proliferating-cell symmetric division rates in sphere and adherent cultures, suggesting functional overlap between the compartments sustaining them. Experiments with normal primary human mammary epithelia, including sorted luminal (MUC1⁺) and basal/myoepithelial (CD10⁺) cells revealed distinct luminal-like, basal-like and mesenchymal entities amongst primary mammospheres. Morphological and colony-forming-cell assay data suggested mammosphere culture may enrich for a luminal progenitor phenotype, or induce reversion/relaxation of the basal/mesenchymal *in vitro* selection occurring with adherent culture. Overall, cell line tumourspheres and primary mammospheres are not homogenous entities enriched for stem cells, suggesting a more cautious approach to interpreting data from these assays and careful consideration of its limitations. Sphere culture may represent an alternative 3-dimensional culture system which rather than universally ‘enriching’ for stem cells, has utility as one of a suite of functional assays that provide a read-out of progenitor activity.

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**Introduction**

Breast cancer research relies heavily on functional assays provided by *in vitro* and *in vivo* models. This includes investigations of the cancer stem cell hypothesis stating that malignant tumours are initiated and maintained by a population of tumor cells that share similar biologic properties to normal adult stem cells. Candidate subpopulations of cancer stem cells (CSCs) can be purified using techniques such as fluorescence-activated cell sorting (FACS), then assayed for stem cell-like properties using *in vitro* clonogenicity, tumoursphere formation and *in vivo* tumourigenicity assays [1,2,3,4,5,6]. These are used to demonstrate key attributes of stem cells: self-renewal and multi-lineage potential, which in the case of CSCs infers the ability to recapitulate the heterogeneity of the original tumour [7,8].

The ability to also expand these subpopulations through other means is seen as an extremely useful tool for breast (cancer) stem cell research. *In vitro* enrichment for normal mammary stem cells...
in non-adherent, serum-free conditions was first reported by Doutt et al [9], varying the method pioneered for neural stem cell cultivation [10]. In these conditions, most cells undergo anoikis whilst rare cells divide and generate spheroid structures - mammospheres. Doutt demonstrated an increased frequency of bi-potent progenitors (defined by the ability to give rise to both luminal and myoepithelial compartments) in spheres compared to the original dissociated tissue, and also that mammosphere immunophenotype was consistent with enrichment for dedifferentiated cells [9].

Sphere formation was then reported in cells from primary breast tumours [3,11], metastases [12] and established cell lines [3,6,12,13,14] and spheres were shown to be enriched for CSC phenotype measured by increased tumour take rate in in vivo xenograft assays. Early reports on the lack or loss of markers of differentiated epithelium (Cytokeratins CK5, CK18, CK19, C14, MUC1, EpCAM and CD10) in spheres [3,9,12] may have promoted the idea of a dedifferentiated state for the entire differentiated mammary epithelium and CSCs). Data also includes characterisation of the morphological and phenotypic differences within primary mammospheres in a basal/luminal-like dichotomy.

Materials and Methods

Propagation and Culture of Tumourspheres from Established Breast Cancer Cell Lines

The adherent growth conditions of the cell lines used in this study are detailed in Table S1. All cell lines were obtained from the ATCC with the exception of SVCT and Hs578T from ECACC, whilst KPL-1 [24] and MCF-12ET [25] were kindly donated Professor Rik Thompson (St. Vincent’s Hospital, Melbourne). All cell lines were authenticated by STR profiling.

To generate tumourspheres, cells were trypsinized from adherent starter cultures with TrypLE (Gibco), quenched in normal growth media, washed three times in large volumes of calcium-magnesium-free PBS to remove as much serum as possible, then passed through a 40 μm cell strainer (BD Falcon). Cell concentrations were determined using the Countess™ automated cell counter (Invitrogen) then seeded in sphere-promoting culture [26] at densities of 1–5×10^3 cells/mL in low-adherent 6-well plates, or 5–10×10^4 cells/mL in low adherent T-75 flasks (Nunc Thermofisher Scientific). NSA media consists of DMEM/F12 (Invitrogen) containing recombinant human epidermal growth factor (EGF; Sigma; 20 ng/mL), recombinant human basic fibroblast growth factor (bFGF; R&D Systems; 10 ng/mL), heparin (Sigma; 4 μg/mL), human or mouse proliferation supplement (NeuroCell™; Stem Cell Technologies; 10%), bovine serum albumin (BSA; Sigma; 0.15%), and penicillin-G-streptomycin solution (Gibco; 1%). Cells were grown at 37°C in a humidified atmosphere containing 5% CO_2_. The overlay method was used for matrigel culture in 96 well plates, whereby cells in suspension were seeded in their appropriate normal growth media containing 4% growth factor reduced matrigel (BD) over 50 μL of 100% matrigel previously set after 30 minutes incubation at 37°C. For clonogenicity experiments, parallel adherent, matrigel and sphere cultures were plated at limiting dilution appropriate for each format (40, 4000 and 800 cells/cm^2 respectively) and resulting clones/structures/spheres were fixed and counted after 7 days. When secondary clonogenicity was performed, spheres were harvested and dissociated as described below, whilst matrigel structures were recovered from the matrix with Dispase (BD) prior to single cell dissociation. Cells were then counted and reseeded in limiting dilution adherent culture as above.
Primary Human Mammary Epithelial Cell Dissociation and Culture

Normal human breast tissue was obtained from consenting women undergoing reduction mammoplasty surgery. Patients gave written consent for the use of their tissue in research and this was approved by the appropriate local Human Research Ethics Committees: the University of Queensland and the Wesley Uniting Hospital. The tissue was dissociated as previously described [27] and the epithelial-rich component was cultured for 7 d in E93 media: F12 media (Gibco), foetal calf serum (FCS; Gibco; 5%), antibiotic/antimycotic (Gibco; 1x), EGF (10 ng/mL), Insulin (Sigma; 5 μg/mL), Hydrocortisone (Sigma; 1 μg/mL) and cholera toxin (Sigma; 100 ng/mL). Cells were then separated by FACS (see below). Sorted cells were seeded in 6-well plates in triplicate at (5×10^4/well) in either adherent (E93 media) or sphere-promoting (Mammocult; Stem Cell Technologies) conditions on Poly-HEMA (Sigma)-coated plates. After 10 d in culture, spheres were harvested for immunohistochemical (IHC) analysis or dissociated for clonogenic assays. Briefly, spheres were collected in 40 μm sieves and then dissociated alongside parallel adherent cultures as above with versene and TrypLE. Resulting single cell suspensions were counted and seeded at 4.5×10^3 cells/well) in either adherent (E93 media) or sphere-promoting (Mammocult; Stem Cell Technologies) conditions for 7 d. After 7 d, cultures were fixed in methanol, stained with Giemsa and colony morphologies assessed by light microscopy.

Sphere-forming Efficiency Assays

Sphere forming capacity (SFC) was assessed at least twice for each of the cell lines listed in Table S1. Cells were seeded in triplicate in low-adhesion 6-well tissue culture plates (2–5×10^3 cells/well) in sphere-promoting conditions. The presence of spheres (3D multicellular structures greater than 40 μm in diameter) was assessed by light microscopy after 7 d and scored based on the number of spheres relative to the number of parent cells seeded: ‘−1’ = no spheres observed, ‘+’ = <=0.01% and ‘++’ =>0.01%. We defined the presence of spheres in a manner similar to described in Maguer-Satta et al., 2011 [29] as clearly three-dimensional spherical-like groups of cells growing as dense, floating and compact clusters, greater than 40 μm in diameter and clearly distinguishable from loose aggregates of cells.

The sphere-forming efficiency (SFE) of six cell lines at passage one was assessed by culturing set numbers of single cells in 50 μL of NSA media in the wells of a 384 well optical bottom plate (Nunc) (5×10^3, 5×10^2, 1×10^3, 5×10^2, 3×10^2 or 1×10^2/well). After 6-7 d in culture, the number of spheres (>40 μm diameter to discriminate from cell clumps, as above) was counted and used to assess the proportion of spheres formed relative to the number of single cells seeded initially.

Sphere Size Determination and Photomicrographs

For sphere sizing, cells were seeded at 1×10^6 in low-adherent T75 culture flasks. 10 random fields/flask were digitally imaged 7 d after seeding, using a digital camera on a CK40 light microscope (Olympus Corporation). Sphere diameters were measured on the long axis using Photoshop CS3 (Adobe). The average size of spheres in the 10 fields or from n = 100 spheres (whichever was greater) was calculated. Measurements were taken in two independent assays and representative results are shown in Fig. 1.

Immunohistochemical Phenotyping of Spheres

After 5 d in culture, spheres were pelleted and fixed in 10% neutral buffered formalin for 1 h before being processed for paraffin embedding. 4 mm Sections were cut for standard Haematoxylin & Eosin (H&E), Periodic Acid Schiff PAS stains or prepared for IHC using different antibodies and antigen retrieval methods (Table 1). The following antigen retrieval methods were used: heat retrieval in a decloaking chamber (Biocare Medical) with 0.001 M Tris/ethylenediaminetetraacetic acid (EDTA) pH 8.8, at 105°C for 15 min or 0.01 M citric acid buffer pH 6.0, at 125°C for 5 min; 0.1% Chymotrypsin in 0.01 M CaCl2+0.05 M Tris buffer, pH 7.8 at 37°C for 10 min. Two detection kits were used: Dako EnVision+ (Dakocytomation) and Vectastain® Universal ABC kit (Vector laboratories) according to the manufacturer’s instructions. Dako HercepTest® kit was used for Her2 staining. Sections were reviewed by two independent observers and described by a qualified pathologist (ACV). At least 2 independent sphere preparations were observed for each antigen.

Determination of Stem Cell Symmetric Division

Ten breast cancer cell lines representing five luminal-like (MCF7, KPL-1, BT-474, SK-BR-3 and T47D), five basal-like (SUM-159-PT, MDA-MB-436, HBL-100 and HS578T all Basal B/claudin-low and Basal A BT-20) cell lines were cultured in adherent and sphere-promoting conditions. Nine lines were cultured at 2.5×10^5 cells/T75 culture flask in triplicate and the total number of cells was calculated every 5 d for 2–8 passages. BT-474 cells were cultured at 1×10^6 cells for sphere-promoting conditions and 8×10^5 cells for adherent conditions in T75 culture flasks in triplicate and total number of cells was calculated every 7 d for 6–8 passages. The rates of long-term proliferating (LTP) cell symmetric division (K_s) was calculated based on fold expansion, using methods previously described [30]. Briefly, fold-expansion represents the cell count at the start of culture divided by the cell count at the end of culture. Symmetric division rate of LTP cells represents the natural log of the fold expansion divided by the time in culture (5 or 7 d). The LTP cell symmetric division reported in this manuscript for KPL-1, MCF7 and BT-474 spheres also appears in the manuscript by Deleyrolle et al. [30], however a different analysis is performed within this manuscript.

Flow Cytometry – Staining and Data Acquisition

Matched adherent and sphere cultures of luminal and Claudin-low cell lines were generated in triplicate (technical replicates) from subconfluent, adherent parent cultures on d0, harvested and dissociated on d7 into single cell suspensions, then concurrently stained with combinations of fluorescent antibody conjugates for simultaneous detection of cell surface markers (Fig. 2). Panel 1: MUC1-FITC (BD), HER2-PE (BD) and the LIVE/DEAD® red cell viability stain (Invitrogen); Panel 2: CD49d-Pacific Blue (Biogenegd), Aldefluor® assay reagent (StemCell Technologies), CD24-PE (BD), LIVE/DEAD® red, AC133-APC (Milteny), EpCAM-PerCP Cy5.5 (BD) and CD44-APC-Cy7 (Biogenegd). Staining with the Aldefluor® assay for detection of ALDH1 activity was done prior to the addition of fluorescent antibodies according to the manufacturer’s instructions. Each Aldefluor-containing sample was prepared in duplicate, and the second negative control sample was immediately quenched in the ALDH1 inhibitor Diethylaminobenzaldehyde (DEAB) as per the manufacturer’s instructions.

Raw fluorescence data was collected on a FACSaria I flow cytometer (Becton Dickinson) using FACSDiva acquisition software (v6.1.3; BD). Particles and dead cells were excluded based on low light scatter and LIVE/DEAD® red positivity. 1×10^5 Events that fell within the live cell gate were collected for each sample. Manual fluorescence compensation was performed on each sample.
A two-stage statistical analysis was then applied to the data to
determine which parameters were significantly and directionally
consistent changes regardless of magnitude, data-
differences between control (adherent) and test (sphere) means
were defined as datapoints 1.5x the interquartile range above the
3rd quartile or below the 1st quartile of the biological and technical
replicates combined, and comprised as significant if at least 2 out of 2 or 3
biological replicates were consistently and significantly altered with
sphere culture. 1) A two-way ANOVA
test was applied: a) to exclude parameters for which there was a
significant treatment experiment interaction (P<0.01, indicating a
lack of reproducibility between biological replicates); and b) to
calculate the statistical significance of consistent changes (‘treat-
ment effects’) considering (data from all experiments where
P<0.05). 2) Whilst the ANOVA procedure is useful for filtering
irreproducible data, it also excluded datapoints where the
differences between treatments and controls for a set of biological
replicates were consistent in direction (i.e. all increases or
decreases), but not in magnitude. Given we were interested in all
directionally consistent changes regardless of magnitude, data-
points with significant treatment experiment interactions were
cross-referenced against two-tailed t-tests for independent biolog-
cal replicates and included as significant if at least 2 out of 2 or 3
biological replicates were consistently and significantly altered with
sphere culture (P<0.05). Data were then finally reviewed, and any
datapoints with borderline significance that were based on
population frequencies <0.25% were removed.

Flow Cytometry - Statistical Analysis

Further data analysis was performed in the R statistical
environment. First, outliers were removed if they represented
obvious deviations from the overall pattern of the data. Outliers
were defined as datapoints 1.5x the interquartile range above the
3rd quartile or below the 1st quartile of the biological and technical
replicates combined, and comprised ~1.2% of the dataset.
Differences between control (adherent) and test (sphere) means
were determined for each biological replicate (minimum 2, usually
3, performed in triplicate).

A two-stage statistical analysis was then applied to the data to
determine which parameters were significantly and directionally
consistently altered with sphere culture. 1) A two-way ANOVA
test was applied: a) to exclude parameters for which there was a
significant treatment experiment interaction (P<0.01, indicating a
lack of reproducibility between biological replicates); and b) to
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sphere culture (P<0.05). Data were then finally reviewed, and any
datapoints with borderline significance that were based on
population frequencies <0.25% were removed.

Primary Breast Epithelial Cell Sorting

Human mammary epithelial cells (hMECs) were harvested by
pre-treatment with versene (Gibco) and TrypLE before washing
and passing through a 40 μm filter. Cells were then adjusted to
1×10^6/mL, and incubated with SYTOX Blue (Molecular Probes;
1:1000), CD10-PE-Cy5, MUC1-FTTC, CD140b-PE, CD31-PE
(all from BD Biosciences at the pre-optimised dilution of 1:100) for
10 min on ice. Live, CD140b-and CD31-negative cells were
sorted for MUC1 and CD10 positivity on a FACS ARIA cell
sorter, collected in Hanks buffered salt solution containing 2%
FCS, then seeded at 1–5×10^3 cells/mL onto poly-HHEMA-coated
plates.

Table 1. IHC methods.

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<th>Dilution</th>
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doi:10.1371/journal.pone.0064388.t001
Results

No Correlation between Tumoursphere-forming Capacity and Breast Cancer Cell Line Subtype, Tumourigenicity or Growth in Matrigel

In order to better understand the biological significance of sphere formation and determine which features might predict for this ability, we tested the abilities of 24 breast cancer and 3 normal breast epithelial cell lines (HBL100, MCF10A, SVCT) to form spheres by seeding at low dilution into non-adherent conditions in serum-free media containing EGF and FGF. Sphere formation was observed in 13/26 cell lines. Comparison to molecular phenotypic data from other studies [31,32,33,34] revealed no clear correlation with sphere formation (Table S1). Non-sphere-forming and sphere-forming cell lines fell in similar proportions amongst luminal-like and basal-like categories [32,34]. Collation of data on cell line in vivo tumourigenicity using various mouse xenograft models [31,35] also failed to stratify cell lines into sphere-forming and non-sphere forming categories, as did morphological phenotypes in matrigel [36] indicating that the relationships between CSC frequency/activity, molecular phenotype, tumourigenicity and sphere-forming efficiency are complex.

Distinct Sphere Morphologies and Immunophenotypes in Basal- and Luminal-like Breast Cancer Cell Lines

Unable to predict sphere-forming ability from the aforementioned properties of adherent cell lines, we decided to more closely examine tumourspheres derived from a smaller panel of breast cancer cell lines, with a view to exploring the idea that tumourspheres are enriched with CSC phenotypes and/or luminal-like and basal-like categories [32,34].
In order to assess sphere formation and characteristics from parental cell-lines of the different major phenotypes, we selected 3 luminal-like and 3 basal-like cell lines, the latter of which are incidentally further subclassified as Basal B [34,37] or claudin-low [38], previously reported to represent a more mesenchymal phenotype that may be enriched with CSC phenotypes. SUM-159-PT and MDA-MB-346 are both frequently assayed basal-like cell lines harbouring RB1 and HRAS mutations respectively [32]. HBL100 was included as an additional basal-like cell-line, clustering on a molecular level with the BasalB [34] or Claudin-low phenotype [38] cell-lines. It is reportedly derived from normal breast tissue however, controversially harbouring a Y function. In order to assess sphere formation and characteristics from parental cell-lines of the different major phenotypes, we selected 3 luminal-like and 3 basal-like cells lines, the latter of which are incidentally further subclassified as Basal B [34,37] or claudin-low [38], previously reported to represent a more mesenchymal phenotype that may be enriched with CSC phenotypes. SUM-159-PT and MDA-MB-346 are both frequently assayed basal-like cell lines harbouring RB1 and HRAS mutations respectively [32]. HBL100 was included as an additional basal-like cell-line, clustering on a molecular level with the BasalB [34] or Claudin-low phenotype [38] cell-lines. It is reportedly derived from normal breast tissue however, controversially harbouring a Y
chromosome [39] which has cast doubt on the cell-line’s true origin. We nonetheless, decided to include this interesting cell line with a view to discerning which sphere features might be phenotype or tissue specific. Amongst the luminal-like cell lines, MCF7 and BT-474 are amongst the most frequently used luminal-like cell lines, both harbouring PIK3A mutations. MCF7 cells exhibit several features of luminal-like breast cancer including retention of ER protein, whilst BT-474 harbour an ERBB2 amplification [32,34]. Sphere size was variable both within and between luminal- and basal-like lines, indicating heterogeneous proliferation rates (Fig. 1A/B). Examination of H&E-stained sections revealed several breast tumoursphere architecture categories (Fig. 1A), somewhat reminiscent of 3D structures formed in matrigel [36,40] (Fig. 1C). HBL100 and SUM-159-PT spheres formed solid structures with irregular edges, comprising cohesive but loosely packed cells. MDA-MB-436 spheres disintegrated upon histological preparation, suggesting unstable or weak inter-cellular adhesion. In contrast to basal-like cell lines, MCF7, KPL-1 and BT-474 spheres comprised tightly packed, cohesive cells within a well-defined border. Whilst BT-474 spheres were spherical solid

![Figure 4. In vitro functional analysis of progenitor cell content in breast cancer cell line tumourspheres. (A) Sphere forming efficiency (SFE) of cell lines through serial sphere passage. Spheres were serially cultivated by dissociating and reseeding cells from each passage at limiting dilution in sphere-promoting conditions. SFE was calculated by counting spheres by light microscopy at d7 of each passage (10–14 replicates per dilution, 4 dilutions/cell line/passage), expressed as a percentage of the original number of cells seeded, then averaged across the dilutions. Bars represent the mean SFE ± SD from 2–3 independent experiments. Two-tailed, unpaired t-tests demonstrated no significant difference in SFE between passage 1 and 5 for any cell line (ns). (B) Correlation between stem cell symmetric division rates of matched adherent and sphere cultures. Ten breast cancer cell lines (MCF7, KPL-1, BT-474, SKBR-3, T47D, SUM-159-PT, MDA-MB-436, HBL100, HS578T and BT-20) were seeded in triplicate at equal densities in adherent or sphere-promoting conditions, and the total number of cells was calculated every 5–7 d for 2–8 passages. Mean fold expansion and the rates of long-term proliferating cell symmetric division (K⁰) were calculated for each culture [30] and plotted for correlation analysis (solid line, linear correlation; dotted lines 95% confidence interval). The relationship between adherent and sphere K⁰ rates was statistically significant (linear correlation analysis (post-test for linear trend); P = 0.0016). (C) Primary clonogenicity of MCF7 and SUM-159-PT cells in different growth formats. Clonogenicity in adherent growth conditions, matrigel overlay and sphere-promoting conditions was calculated as the number of colonies (adherent or non-adherent) that grew after 7 d, as a percentage of cells seeded. Data shown are means ± SE, representative of at least three separate experiments performed in triplicate. P values in C and D were generated using two-tailed, unpaired t-tests. doi:10.1371/journal.pone.0064388.g004]
masses, a proportion of KPL-1 and MCF7 spheres were hollow. KPL-1 structures were consistently of irregular shape and contained inner cell-like lumina reminiscent of papillary hyperplasia (Fig. 1A). MCF7 spheres exhibited both solid symmetrical and asymmetrical structures with lumina. Mitoses and apoptotic cells were prominent in spheres from all six lines (data not shown).

To investigate molecular heterogeneity and differentiation states between and within the cell line spheres, we performed immunohistochemical (IHC) analysis. Spheres from basal- and luminal-like lines exhibited distinct basal/mesenchymal and luminal IHC profiles respectively (Fig. 1D, Fig. S1). Basal-like spheres were homogeneously negative for CK19, CK14, CK5 (Fig. S2), oestrogen receptor (ER), EpCAM, MUC1 and E-cadherin, and positive for basal markers CD44 and EGFR, and the mesenchymal marker vimentin [32]. Overall, luminal-like spheres demonstrated fidelity to the differentiated luminal phenotype (ER+, CK19+, E-cadherin+, EpCAM+, MUC1+, vimentin+ and EGFR+), although unlike basal-like spheres, heterogeneity was observed both between and within luminal-like spheres for ER, HER2, EpCAM, CD44 and MUC1. CD44 expression was concentrated in patches (Fig. 1Dxvii). Similarly, MUC1 showed mostly diffuse cytoplasmic staining, although prominent ‘apical’ or membranous staining was sometimes observed for KPL-1 and MCF7 (Fig. S1). Overall, sphere morphologies and phenotypes reflected the adherent parent lines and did not exhibit gross molecular dedifferentiation as suggested by Ponti [3], although luminal-like spheres exhibited some molecular heterogeneity. Both basal- and luminal-like spheres were capable of secreting their own Laminin extracellular matrix (Fig. 1Dxvii/xviii, Fig. S1k).

Breast Cancer Cell Line Spheres are not Universally Enriched for Markers of Stem Cell Activity

Subsequent to the discovery that the combination of two markers, CD44 and CD24, can define and enrich for an important stem cell population in breast tumours by flow cytometry [1], others demonstrated that the addition of a third marker to this combination could further purify cells with different functional characteristics, namely EpCAM+/CD44+/CD24− [13] and Aldefluor+/CD44+/CD24− [41]. HER2 [4,17], CD49f [6], AC133 [14] have also been shown to define or influence the activity of breast cancer stem cell populations and MUC1, in addition to delineating the mature luminal subpopulation of normal cells, has been shown to be overexpressed in a majority of breast cancers [42]. We therefore designed a flow cytometry protocol to simultaneously detect six markers previously shown to be important in delineating functional mammary epithelial cell subpopulations (Fig. 2A), enabling detection of a large number of possible marker combinations and subpopulations. We hypothesised that if spheres were enriched for CSC activity or any particular progenitor subpopulation, previously reported CSC markers, or novel marker combination phenotypes, would be more frequent within sphere preparations, which if rare could be difficult to quantitate using standard IHC analyses (Fig. 1D). To assess whether molecularly-defined subpopulations are enriched with sphere culture, we applied two fluorescent antibody conjugate panels (comprising MUC1, HER2, CD49f, CD24, CD44, AC133, EpCAM and Aldefluor®, an enzyme assay that gives a read-out of ALDH1 activity) to screen for any changes in subpopulation frequencies between parallel, 7-day sphere and adherent cell line cultures (Fig. 2). The data are presented as differences in subpopulation frequencies that were reproducible between biological replicate experiments, and statistically significant (Fig. 3; representative raw data in Figs S3 and S4).

Examination of single marker positivity between sphere and adherent cells revealed no consistent changes amongst the 6 cell lines, or across molecular subtypes (Fig. 3A), although cell line-specific changes were observed, reinforcing the idea that spheres are molecularly heterogeneous. ALDH1 activity was enriched in MDA-MB-436 and MCF7 spheres, while CD49f was reduced in spheres for SUM-159-PT and MCF7 (in contrast to a previous report [6]). AC133 positivity was enriched in 3 of the cell lines tested (HBL100, and highly significantly enriched in MCF7 and BT-474 spheres (P<0.0001, Fig. 3A dashed box; Fig. S3B)). The significance of this is unclear, but it is noteworthy that AC133 positivity has been associated with a phenotypic shift from bipotency to a committed luminal progenitor state in normal primary human mammary epithelia [43]. Moreover, others have shown AC133 is associated with SLUG expression in primary breast tumourspheres [44], and SLUG is associated with accumulation of luminal progenitor cells and defective luminal lineage commitment in human breast tumours [45].

Examination of marker combinations also revealed variability, with no consistent changes observed across cell lines or within subtypes. We did not observe consistent increases in the frequency of CD44+/CD24− cells (nor with further stratification with EpCAM or ALDH1) that we hypothesised may be more frequent in spheres based on previous reports (Fig. 3B). We did observe frequent changes in CD49f/CD24 distribution with sphere culture, with significant loss of CD49f+/CD24− or CD49f−/CD24+ cells in 4/6 lines tested (SUM-159-PT, MDA-MB-436, MCF7 and KPL-1; P<0.05, Fig. 3B dashed box; Fig. S4B). Given others have shown that CD49f/CD24 distribution can reflect broader states of differentiation defined by multiple biomarkers as well as morphology [46], these data suggest that culturing cell lines as spheres may alter their differentiation programs. Of technical importance, we often observed higher levels of autofluorescence in spheres compared to adherent which might erroneously inflate sphere expression levels if not considered by running comprehensive controls and in threshold and gate application. In order to confirm our observations in BT-474 and SUM-159-PT (Figs S2C and S3B; CD49f), we independently validated these by switching CD49f to an alternative, unaffected fluorochrome (data not shown).

In addition to these findings, we did observe cell-line specific changes for several marker combinations, including spheres from HBL100 (AC133+/CD49f−, MDA-MB-436 (CD44+/CD24−/CD49f−), and BT-474 (CD44+/CD24+/AC133+ and CD49f−/CD24+/AC133+), which were not only reproducibly observed between biological replicates, but highly statistically significant (P<0.0001) and occurred independently of changes in the respective single markers (Fig. 3A). Overall our observations indicate, with the markers we have examined, that there are no consistent global changes in breast cancer cell line spheres including putative cancer stem cell combinations, but that each cell line may undergo its own individual increases or decreases in marker expression when cultured in this format.

In vitro Functional Analysis of Breast Cancer Cell Line Tumoursphere Progenitor Cell Content Compared to Adherent Cells and Matrigel Structures

Since morphologic and immunophenotypic analyses suggested that spheres are not consistently enriched with particular immuno-phenotypes, we explored whether they could be enriched for CSC activity using in vitro assays of self-renewal and clonogenicity. Serial sphere passage has been reported to increase sphere-forming efficiency (SFE) in MCF7 cells [6]. We reasoned that if this was a general phenomenon in sphere-forming lines, comparison of early
with late passage spheres could be used to identify new CSC markers. We determined SFE in the 6 cell line panel over 5 serial passages, dissociating spheres and reseeding single cells after 7 days. SFEs were similar at first passage (3.0–4.4%, 7% for HBL100), and surprisingly, this was unchanged over serial passage in all lines tested (Fig. 4A), indicating there was no enrichment with sphere-initiating cells, and that comparing early and late passage spheres was unlikely to be a useful approach for studying CSC phenotype or function.

In order to determine whether the cell-lines grew at different rates and therefore exhibit different progenitor frequencies and/or self-renewal rates in spheres or in traditional adherent culture, we

Figure 5. Mammospheres derived from MUC1+ or CD10+ progenitors from reduction mammoplasties comprise hollow and solid structures consistent with luminal- and myoepithelial-like morphologies. (A) Strategy for isolating luminal and myoepithelial progenitor-enriched subpopulations from reduction mammoplasty tissue. Tissues were physically and enzymatically processed to epithelial-rich, single cell suspensions, then cultured for 7d in mammary epithelial growth medium to generate enough cells for fluorescence-activated cell sorting (FACS) and sphere culture. Primary cultures were stained with fluorescent antibody conjugates (MUC1-FITC, CD10-PE-Cy5), the Sytox® Blue viability stain and ‘Lineage’ cocktail (Lin': CD140b-PE, CD45-PE, CD31-PE; markers of stromal fibroblasts, leukocytes and endothelia respectively). CD10 and MUC1-sorted cells were placed in sphere-promoting culture for 10d before analysis. (B) Gating strategy for enrichment of luminal- and myoepithelial-like progenitor cells. Acellular particles and dead cells were excluded based on low light scatter and Sytox® blue positivity. Non-epithelial, PE+ cells were also excluded, then MUC1+ and CD10+ cells were collected for sphere culture. (C) Light microscope images (i,iii) and H&E histological sections (ii,iv–vii) of spheres generated from CD10+ or MUC1+ primary breast epithelial cells. Open arrows, MUC1+-derived spheres often had limited lateral connections giving a petal-like appearance. Solid arrows, shows single cell with signet ring secretory morphology. (D) Immunophenotypic analysis of MUC1+ and CD10+ progenitor-derived primary breast mammospheres. Spheres were generated as described in A/B. Representative images from immunohistochemical analysis of the indicated antigens on FFPE preparations of spheres are shown. Images taken at 200x magnification. Scale bar 100 μm. Black arrows indicate apical membranous staining of E-cadherin and EGFR in luminal-like spheres. Red arrows indicate the mesenchymal-like spheres found rarely amongst the dominant structures formed in both CD10+ and MUC1+ sorted populations.

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compared the fold expansion rates of 10 cell lines grown in both formats in parallel. After applying a mathematic model that uses fold expansion over serial passage to calculate the long-term proliferating cell symmetric division rate ($K_0$) [30], we observed a significant correlation between matched sphere and adherent cultures ($P=0.0016$; Fig. 4B). Essentially this shows growth rate is inherent to the cell line and not the culture format. This raises the possibility that the same progenitor populations could sustain sphere and adherent cultures, but that the symmetric division rate in spheres is restricted by physical limitations (lack of growth factors, matrix attachment and/or biophysical size restrictions). This casts further doubt on the notion of spheres containing an enriched population of cells with different growth characteristics.

We then assessed the relative frequencies of sphere- and matrigel structure-initiating cells compared to standard adherent conditions by calculating clonogenicity (% of cells capable of initiating new clonal growth) after seeding cells at clonal density in the different growth conditions (Fig. 4C). We reasoned this would indicate the frequency of colony forming cells in each culture format. Interestingly, MCF7 clonogenicity was highest in matrigel (43%) compared to adherent (24%), whilst SUM-159-PT was similar (37%) in matrigel when compared to adherent (45%). As expected, clonogenicity in sphere-promoting conditions (SFE) was much lower: 4% for MCF7 and 1% for SUM-159-PT. This suggests that only rare cells are capable of generating full clones in these conditions.

Given the published evidence of increased tumourigenicity from spheres compared to adherent cultures, but having failed to observe any enrichment with self-renewing, sphere-forming cells (Fig. 4A), we decided to analyse enrichment for other progenitor or colony-forming cells. We therefore compared the secondary clonogenicity rates of spheres and matrigel structures, dissociating them and reseeding equal numbers of cells at clonal density in adherent conditions. Surprisingly, both spheres and matrigel structures exhibited secondary cloning rates comparable to primary clonogenicity in adherent conditions (Fig. 4D compared to 4C), indicating that spheres are no more enriched with adherent culture-competent progenitor cells than cells maintained in adherent or matrigel cultures. One interpretation of this is that progenitor cells reside in these structures at similar frequencies.

**Multiple Morphologically and Phenotypically Distinct Entities in Normal Primary Mammary Epithelial Mammosphere Preparations and Enrichment in FACS-sorted Sub-populations**

Having observed heterogeneity in tumourspheres derived from breast cancer cell lines, we decided to investigate whether primary normal human mammary epithelial cell (hMEC) mammospheres also exhibit such diversity or whether they are single entities that can be pooled for molecular analysis. After preliminary observations revealing the presence of solid and hollow mammospheres from fresh dissociations of reduction mammoplasty tissue (Fig. S5), we compared the phenotypes of spheres derived from sorted hMECs grown as spheres or adherent cultures from 4 different patients (Fig. 6B), indicating sphere culture does not enrich for progenitor activity compared to adherent culture. However, we did observe a significant increase in the ratio of luminal to myo/mixed colonies in MUC1+ subpopulation of primary cells. We observed no overall difference in the clonogenicity of MUC1+ sorted hMECs grown as spheres or adherent cultures from 4 different patients (Fig. 6C), suggesting selection of progenitors already locked into a luminal fate or promotion of a luminal lineage differentiation axis. Alternatively, this luminal bias could occur through relaxation or reversion of the adherent in vitro selection of basal/mesenchymal states that is thought to occur during the generation of breast cancer and normal breast cell lines [46], providing there is pre-existing luminal progenitor potential in the starting culture.

**Discussion**

This study has comprehensively characterised, for the first time, different types (by both morphological and molecular definition) of spheres derived from a panel of 26 breast cancer cell lines and primary normal hMECs. The results conclusively demonstrate that spheres are not homogeneous structures enriched with undifferentiated cells, but rather comprise a range of morphologically distinct entities displaying inter- and intra-sphere molecular heterogeneity, including variable expression of markers of differentiated mammary epithelia. Importantly, we show for the first time that this heterogeneity extends to primary normal...
mammosphere cultures. MUC1 cultures. (C) Comparison of luminal and myo/bipotent progenitor cell clonogenicity of spheres and parallel adherent cultures. Data are from four biological replicates (specimens from 4 patients), each performed in triplicate. Statistical tests used were paired, two-tailed students t-tests ($P$ values indicated; $n_s$ = not significant). doi:10.1371/journal.pone.0064388.g006

Heterogeneity between and within spheres has very important implications, both practical and conceptual. Firstly, sphere preparations contain mixed structures that would likely ambigu- date or dilute observations made from pooled, bulk cultures. The data demonstrate that analysis of bulk primary mammosphere cultures in particular, may be no more informative than analyses of a mixture of all primary hMEC types. Interestingly, this dilemma may also apply to more ‘clonally regarded’ cancer cell lines, as Matilainen et al. recently generated two distinct sphere phenotypes from 4T1 cells [23]. Second, the detection of multiple sphere types implies the existence of distinct sphere-initiating cell types. This alerts us to the existence of multiple functional populations within any pool of cells, several of which may be sphere-formers. It also highlights that the ways in which we define functional hMEC subpopulations are limited by our understanding of the biological significance of the various assays originally used for their identification. For example, a particular subpopulation, defined by a defined combination of molecular markers, may show stem cell activity in some assays, and not in others.

The lack of correlation between sphere-forming capacity and known features across our large cell line panel (Table S1), and the variability in expression of key molecular markers observed by comprehensive, multi-parametric flow cytometry profiling (Fig. 3) suggest that we are unlikely to arrive at a universal understanding (or indeed a common set of markers) of the biological significance of sphere formation and its relevance to stem cell phenotype and function. Rather, each cell line and its various subpopulations are unique. These interpretations are supported by work with MCF7 spheres demonstrating a lack of correlation between CD44$^+$/CD24$^-$ phenotype and sphere formation, tumourigenicity and radiation resistance, with data suggesting separate but sometimes overlapping cell populations [15]. In our multiparametric flow cytometry experiments, we were unfortunately limited in our choice of markers by the commercial availability of fluorescent conjugated antibodies. Further experiments, however, including other markers such as Thy-1 and CD10, recently shown to be important for purifying particular mesenchymal-like stem cell populations in breast cells [48], may be insightful particularly for delineating for mesenchymal and basal-like subpopulations in multiple cell lines.

It is possible that previous reports of positive correlations between stem cell marker frequency, sphere formation, radiation- and chemo-resistance and in vivo tumourigenicity may have lead to some overgenerous definitions of CSCs and the suitability of the sphere-assay for functional investigation [3,4,6,18]. Several recent reviews also challenge the biological significance of the assay, and are important reading for any researcher culturing spheres [20,50]. In a similar vein, Visvader and Lindeman have cautioned, “…the defining characteristics of these different spheres and their relationship with normal stem cells have been unclear, causing over-interpretation of results… it remains to be determined whether non-adherent spheres selectively enrich for CSCs” [51].
Conclusions

In summary, we favour the view that sphere culture could be a system that selects progenitor cells in a phenotypic state permitting growth-factor-independence and anoikis resistance, with utility for modelling aspects of breast cancer or mammary differentiation. Extreme caution must be taken, however, in over-interpreting results when the biological significance of the assay is still poorly understood. Our data counters the notions that spheres are single entities and enriched for stem cells. Instead we demonstrate extensive heterogeneity between spheres and the cells that comprise them, implying more complex relationships between sphere formation and other common methods of defining stemness. Studying spheres themselves to understand the biology of normal or CSCs is probably no more informative than studying heterogeneous mammary gland or tumour tissue.

Supporting Information

Figure S1 Immunohistochemical analysis of breast cancer cell line spheres. Immunohistochemical analysis of indicated antigens on FFPE preparations of spheres from three basal- and three luminal-like cell lines. Images were taken at 200x magnification, unless where indicated by black triangle at 400x magnification. Scale bar represents 100 μm. Grey arrowheads indicate areas of HER2 immuno-positivity. Black arrowheads indicate intermittent laminin1/2+ and PAS+ cells in luminal cell spheres. White arrowheads indicate bright PAS staining along the edge of MCF7 and KPL-1 spheres. (PDF)

Figure S2 Immunohistochemical analysis of CK5 and CK14 in basal breast cancer cell line spheres. Immunohistochemical analysis of indicated antigens on FFPE preparations of spheres from three basal-luminal-like cell lines. Images were taken at 200x magnification. Scale bar represents 50 μm. (PDF)

Figure S3 Changes in the activity of ALDH1 (A) and expression of EpCAM, CD133 (AC133) (B) and CD49f (C) with sphere culture. Adherent and sphere cultures were dissociated, stained with fluorescent antibody conjugates and analysed as described in Fig. 2. Representative data are depicted using dot or contour plots. Subpopulation frequencies shown represent the percentage of live cells. Quadrant gates were placed at the threshold of autofluorescence for respective adherent or sphere unstained control samples. (PDF)

Figure S4 Changes in cell line differentiation states with sphere culture. Adherent and sphere cultures were dissociated, stained with fluorescent antibody conjugates and analysed as described in Fig. 2. (A) Changes in CD44+/CD24- phenotypes with sphere culture. Representative data are depicted using contour plots. Subpopulation frequencies shown represent the percentage of live cells. Quadrant gates were placed at the threshold of autofluorescence for respective adherent or sphere unstained control samples. For KPL-1, the red circle indicates a consistent gain of a CD44+/CD24- subpopulation (not statistically significant by statistical analysis of quadrant gates but visually obvious). (B) Changes in CD49f+/CD24+/EpCAM distributions with sphere culture. Where the CD49f+/CD24+ distribution of EpCAM+ cells differed between adherent and sphere cultures, pie charts indicate the relative proportions of EpCAM+ and EpCAM- cells in each quadrant. 1x10^5 events displayed on all plots. Red and blue quadrant colouring is transposed from Fig. 3 to indicate subpopulation frequencies that were consistent
and statistically significant across biological replicates (red, increased in spheres compared to matched adherent cultures; blue, decreased in spheres).

**Figure S5** Light micrographs of hollow and solid spheres formed from fresh dissociations of normal human breast tissue. Images were taken at 100x magnification after 10 days in culture.

**Table S1** Breast cancer cell lines: growth media and sphere-forming capacity. Cell lines were cultured as monolayers in the media indicated. Sphere-forming capacity (SFC) was then determined in triplicate on at least 2 occasions by seeding a standardised number of cells in sphere-promoting conditions (see materials and methods), then counting the number of spheres at 7 d relative to the number of parent cells seeded: ‘−’ = no spheres observed, ‘+’ = <0.01% and ‘++’ >0.01%. SFC was then correlated with adherent growth media, and published data (intrinsic molecular subtypes, tumourigenicity in mouse xenograft assays and 3D in vitro morphology in laminin-rich extracellular matrix (hECM)), however we found no obvious association of any of these parameters with in vitro tumoursphere-forming capacity (SFC).

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**Author Contributions**

Conceived and designed the experiments: CES BM JS SRL JAL. Performed the experiments: CES BM JS PK LR MAA. Analyzed the data: CES BM JS ACV LW DS PS. Contributed reagents/materials/analysis tools: BAR CWS. Wrote the paper: CES BM JS. Securing Funding: PS SLR JAL BR.

**References**


