# CLINICAL SIGNIFICANCE OF BREAST CANCER STEM CELLS

BY KAY N. DIAS B.Sc.

A Thesis Submitted to the School of Graduate Studies in Partial Fulfillment of the Requirements for the Degree Master of Science

McMaster University MASTER OF SCIENCE (2014) Hamilton, Ontario
(Medical Sciences)

TITLE: Clinical significance of breast cancer stem cells

AUTHOR: Kay Dias, B.Sc. (McMaster University)

SUPERVISOR: Dr. Anita Bane

NUMBER OF PAGES: xiii, 90

## ABSTRACT

Tumour initiation and progression is thought to be driven by a small population of tumor initiating cells (TICs) or cancer stem cells (CSCs), which have the capacity to migrate and cause metastases and contribute to tumour relapse. These cells possess properties that are similar to those of normal tissue stem cells, which include the capacity to undergo self-renewal as well as the capacity to give rise to more differentiated progenitor cells, which comprise the bulk of the tumour cell population. Thus far, the clinical significance of these cells in breast cancers has not been extensively explored with regard to their relationship with tumour pathology or patient survival. In this thesis we evaluate the presence of these cells in terms of clinicopathological tumour characteristics and patient outcome, as well as assess potential markers of breast CSCs for prognostic significance. Through the quantification of breast CSCs in primary breast tumours using *in vivo* xenografts assays we show that their presence correlates with aggressive tumour characteristics. In addition, we propose that markers of breast CSCs may differ based on the molecular subtype of the tumour, and that these markers have prognostic significance in patients.

**ACKNOWLEDGEMENTS**

I would like to acknowledge and thank my M.Sc. supervisor Dr. Anita Bane for providing me with the opportunity to complete my graduate studies under her supervision. I am extremely grateful for your mentorship, encouragement and support during my time in your lab.

I would like to thank my committee members Dr. John Hassell for his guidance and generous use of his lab during my project, and Dr. Jonathan Bramson for his insight and helpful feedback.

I would like to acknowledge the members of the Bane and Hassell labs for their continuing support, especially Craig Aarts for his expertise with mouse work, Anna Dvorkin-Gheva for her help with bioinformatics, Greg Pond for his generous help with biostatistics, and Jess Cockburn for her mentoring and friendship. I would also like to thank Ying, Tanja, Adele, Bonnie, Robin, Kayla, Melissa, Will, Natallia, Brian and Amy for their assistance in the lab, helpful discussions, and friendship.

Finally, I would like to thank my family, for their patience, encouragement and love.

## TABLE OF CONTENTS

## LIST OF TABLES & FIGURES

## LIST OF ABBREVIATIONS

| | |
|---|---|
| AHWBI | Accelerated hypofractionated whole breast irradiation |
| ALDH1 | Aldehyde dehydrogenase 1 |
| BMP | Bone morphogenic protein |
| Brca1 | Breast cancer 1 |
| CI95 | 95% confidence interval |
| CK5 | Cytokeratin 5 |
| CSC | Cancer Stem Cell |
| CXCL12 | Chemokine (C-X-C motif) ligand 12 |
| CXCR4 | C-X-C chemokine receptor type 4 |
| DAB | Diaminobenzidine |
| DFS | Disease-free survival |
| DMEM | Dulbecco's Modified Eagle Medium |
| DMSO | Dimethyl sulfoxide |
| ECM | Extracellular matrix |
| EGF | Epidermal growth factor |
| EGFR | Epidermal growth factor receptor |
| ELDA | Extreme limiting dilution analysis |
| EMT | Epithelial to mesenchymal transition |
| EpCAM | Epithelial cell adhesion molecule |

| | |
|---|---|
| ER | Estrogen receptor |
| ESA | Epithelial surface antigen |
| FBS | Fetal bovine serum |
| FDA | Food and drug administration |
| FFPE | Formalin-fixed paraffin embedded |
| FGF | Fibroblast growth factor |
| FISH | Fluorescence in situ hybridization |
| H&E | Hemotoxylin and Eosin |
| HBSS | Hank's balanced salt solution |
| HER2 | Human epidermal growth factor receptor 2 |
| IHC | Immunohistochemistry |
| ITGA6 | Integrin alpha 6 (also known as CD49f) |
| KLF4 | Kruppel-like factor 4 |
| LDCT | Limiting dilution cell transplant |
| LR | Local recurrence |
| Lum A | Luminal A |
| Lum B | Luminal B |
| mRNA | Messenger RNA |
| NOD/SCID | Non-obese diabetic/severe combined immunodeficient |
| NSG | NOD/SCID IL2R$\gamma^{null}$ |

| | |
|---|---|
| OCT4 | Octamer binding transcription factor 4 |
| OS | Overall survival |
| PBS | Phosphate buffered saline |
| PDX | Patient-derived xenograft |
| PR | Progesterone receptor |
| qRT-PCR | Quantitative real time polymerase chain reaction |
| REB | Research ethics board |
| RNA | Ribonucleic acid |
| SFA | Sphere forming assay |
| SFF | Sphere-forming frequency |
| SOX2 | Sex determining region Y-box 2 |
| TEDTA | Trypsin-Ethylenediaminetetraacetic acid |
| TGFβ | Transforming growth factor beta |
| TIC | Tumour Initiating Cell |
| TMA | Tissue microarray |
| TN | Triple Negative |

# INTRODUCTION

## 1.1 The Cancer Stem Cell Theory

The cancer stem cell model of cancer progression proposes that a tumour originates from a small population of tumor initiating cells, or cancer stem cells (CSCs) (Clevers, 2011; Dalerba, Cho, & Clarke, 2007). This population of cells is thought to drive tumour progression with the capacity to migrate and cause metastases, contributing to tumour relapse. Additionally, this subpopulation has been demonstrated to be resistant to chemotherapy and radiation therapy (Dean, Fojo, & Bates, 2005). CSCs possess properties that are similar to those of normal tissue stem cells, which include the capacity to self-renew as well as the capacity to give rise to more differentiated progenitor cells which comprise the bulk of the tumour cell population (Wicha, Liu, & Dontu, 2006).

The origin of the cancer stem cell is a much debated topic; thus far there is no consensus as to whether the origin of these cells is due to the transformation of a normal tissue stem cell, or whether a CSC represents a transformed cell which has acquired features of a stem cell (Visvader, 2011). This is further complicated by the phenotypic plasticity between CSCs and non-CSCs, resulting in a more dynamic CSC model where more differentiated cells within the tumour can undergo a dedifferentiation process and acquire CSC characteristics under certain conditions, namely signals from the stem cell niche (Supplementary Figure 1)

(Marjanovic, Weinberg, & Chaffer, 2013; Vermeulen, de Sousa e Melo, Richel, & Medema, 2012). The stem cell niche is a cellular microenvironment that is comprised of stroma, extracellular matrix (ECM), cytokines and growth factors, which provide signals for the maintenance of the stem cell state (Sugihara & Saya, 2013).

In contrast, the stochastic model of cancer progression proposes that somatic cells acquire the mutations necessary for tumour formation and each cell of the tumour retains the capacity to propagate the malignant phenotype, and can seed and establish a metastatic colony (Supplementary figure 2) (Greaves & Maley, 2012; Shackleton, Quintana, Fearon, & Morrison, 2009).

This has important implications for cancer therapeutics (Supplementary figure 3). Current therapies are predominantly aimed at reducing the more rapidly dividing cells that comprise the bulk of the tumour, but may not be effective against the small population of CSCs. Treatments that selectively target the CSC population, in addition to traditional therapies would potentially be more effective at reducing the risk for recurrence and metastases (Tirino et al., 2013; Wicha et al., 2006). However, under the dynamic model of the CSC theory, more differentiated cells could de-differentiate into CSCs and contribute to relapse, suggesting that the stem cell niche should also be considered for potential targeted therapy to achieve durable long term remission (Sugihara & Saya, 2013; Vermeulen et al., 2012)

**1.2 Assays used to study cancer stem cells**

To examine whether CSCs can function as prognostic or predictive biomarkers to aid in the management of cancers patients, we must first be able to identify and quantify this population of cells using robust and accurate means. Assays for the identification and quantification of CSCs include: the *in vivo* limiting dilution xenograft assay and the *in vitro* sphere forming assay (SFA).

Currently, the gold standard test for quantifying the frequency of stem cells in a solid tumour is the *in vivo* limiting dilution cell transplantation (LDCT) assay, which involves injecting various dilutions (e.g. $1X10^5$, $1X10^4$, $1X10^3$ and $1X10^2$) of human tumour cells into immunocompromised mice (e.g. NOD/SCID IL2R$\gamma^{null}$ (NSG)) and monitoring tumour growth at each dilution at different time points (Clarke et al., 2006). This method facilitates the quantification of functional cancer stem cells or tumour initiating cells, and is a 'read out' of both self-renewal capability, through serial transplantation of these tumours, and differentiation capability by examining the cellular heterogeneity of the xenografted tumours.

The technical difficulty, expense and time required for LDCT assays limits the wide spread adoption of this assay to quantify CSCs. An *in vitro* assay for the identification of CSCs is the sphere forming assay which involves growing cells under non-adherent conditions in defined media containing EGF and FGF. Such stringent conditions reportedly only allow for the growth of CSCs which

proliferate to form spheres (Dontu et al., 2003; Reynolds & Weiss, 1992). Using this assay, the number of CSCs or sphere forming cells can be quantified by counting the number of spheres that are formed after plating a defined number of single cells to calculate a sphere forming frequency (SFF). This assay assumes that each sphere is derived from a single CSC or tumour initiating cell (TIC). The advantages of SFAs over LCDT assays include ease of assay performance and the modest investment in time and money to conduct these forms of experiments. Sphere forming assays however are not without their limitations; most notably, spheres are prone to aggregation and thus each sphere may not be derived from a single CSC or TIC (Pastrana, Silva-Vargas, & Doetsch, 2011). However, since the assays are technically easier and more cost effective to conduct, they are a useful tool to estimate stem cell frequency of tumours and could be used to identify putative markers of CSCs.

### 1.3 Identification of CSCs

Cancer stem cells were first identified in the hematopoietic system by Bonnet and Dick (1997), where different subtypes of acute myeloid leukemia were engrafted into immunocompromised mice. Engraftment was only successful from a subpopulation of $CD34^{+}CD38^{-}$ expressing cells. In addition, these cells were found to be extremely rare, in the order of one out of a million (Bonnet & Dick, 1997). Since then potential CSC populations have been found in a variety

of different solid tumours, including breast, glioblastoma, melanoma, lung, prostate, ovarian, gastric and colon cancer (Al-Hajj, Wicha, Benito-Hernandez, Morrison, & Clarke, 2003; Bapat, Koppikar, & Kurrey, 2005; Collins, Berry, Hyde, Stower, & Maitland, 2005; Fang et al., 2005; Ricci-Vitiani et al., 2007; S. K. Singh et al., 2003; Takaishi et al., 2009).

### 1.4 Identification of Breast CSCs through LCDT assays

Al-Hajj et al. (2003) identified a subset of breast tumourigenic cells from eight pleural effusions and one primary breast tumour that were $CD44^{+}CD24^{-/low}Lineage^{-}$. CD44 and CD24 are cell surface glycoproteins, primarily involved in cell adhesion and migration through their interaction with other cells and the extracellular matrix including collagen, laminin and fibronectin (Aruffo, Stamenkovic, Melnick, Underhill, & Seed, 1990; Fogel et al., 1999). This subpopulation of cells was able to form tumours in immunocompromised mice when as few as 1000 cells were injected, whereas cells with other phenotypes failed to form tumours even when injected with tens of thousands of cells. Further enrichment of tumourigenic cells was done by isolating the $ESA^{+}$ (epithelial surface antigen, also known as epithelial cell adhesion molecule or EpCAM) fraction of the $CD44^{+}CD24^{-/low}$ subpopulation and as few as 200 $ESA^{+}CD44^{+}CD24^{-/low}Lineage^{-}$ were able to form tumours in mice. These cells were also able to recapitulate the phenotypic heterogeneity from the initial tumour

and were also serially passaged through tumour formation in mice (Al-Hajj et al., 2003). This is illustrative of the central features of CSCs with respect to their ability to self renew and generate tumours comprised of heterogeneous cells.

A variety of other breast CSC markers have been proposed. Ginestier et al. (2007) identified another subset of cells that expressed high levels of aldehyde dehydrogenase 1 (ALDH1), a detoxifying enzyme that is responsible for the detoxification of intracellular aldehydes. The ALDEFLUOR assay, which measures ADLH enzyme activity using a fluorescent permeable substrate which is retained in ALDH positive cells and assessed by flow cytometry, was used to sort cells from patient-derived xenografts of breast tumours into ALDH positive and negative fractions and injected into immunocompromised mice. As few as 500 ALDEFLUOR-positive cells were able to generate tumours and be serially passaged, whereas 50,000 ALDEFLUOR- negative cells were required to generate even limited tumour growth (Ginestier et al., 2007).

### 1.5 Identification of Markers of Breast CSC markers from Sphere Forming Assays

Studies have also shown that sphere forming cells, usually from breast cancer cell lines, provide information about potential CSC markers, through the identification of cell surface proteins as well as the identification of genes involved in stem cell processes through gene expression studies.

Wright et al. (2008) found that a population of *Brca1* cells positive for CD133 were found to have a similar stem cell phenotype to that of $CD44^+/CD24^-$ cells, even though there was no overlap between these populations in *Brca1* cell lines. CD133, also known as prominin 1, is a five transmembrane domain cell surface glycoprotein protein that is typically expressed on different types of human and murine stem cells (Shmelkov, St Clair, Lyden, & Rafii, 2005). Both $CD44^+/CD24^-$ and $CD133^+$ cell populations were able to repopulate the parent cell fractions and were resistant to chemotherapy *in vitro* and were also able to initiate tumours in immunocompromised mice when as few as 50 cells were injected (Wright et al., 2008). When cells were sorted for $CD44^+/CD24^-$ and $CD133^+$ cell populations and analysed for the expression of other genes characteristic of stem cells (e.g. Oct4 and Numb), there was an elevated expression of these genes in both cell populations, even though they comprise independent groups of cells (Wright et al., 2008).

### 1.6 Markers of Normal Mammary Stem Cells and Embryonic Stem Cells

In addition to the cell surface markers already identified for breast CSCs, other markers including cytokeratin 5 (CK5) and CD49f (also known as integrin $\alpha 6$) have been proposed as potential markers of CSCs (Boecker & Buerger, 2003; Horwitz, Dye, Harrell, Kabos, & Sartorius, 2008; Lim et al., 2009). CK5 is a type II keratin protein expressed in basal epithelium cells and is a characteristic marker

of basal-like breast cancer, and has been found to be expressed in a small tumourigenic population of CD44 positive, estrogen receptor (ER) and progesterone receptor (PR) negative cells in ER positive tumours (Horwitz et al., 2008). Cd49f is a transmembrane protein that mediates interactions between adhesion molecules on adjacent cells or the extracellular matrix and is part of focal adhesion complexes that have various roles in cell biology including cell migration, differentiation and apoptosis and has been found to be a marker of mammary progenitor cells (Eirew et al., 2008; Lim et al., 2009).

Embryonic stem cell and mammary epithelial stem cell markers have been implicated in the maintenance of breast CSCs. NANOG, SOX2, OCT4 and KLF4 are embryonic stem cell transcription factors that are responsible for the regulation of pluripotency and the maintenance of the undifferentiated state (Yamanaka, 2008). While these factors are not highly expressed in the normal adult breast, studies have found an increase in their expression in early stage breast tumours including their expression in mammospheres and tumourspheres, and have implicated their role in the self-renewal of CSC in breast tumours, (Ezeh, Turek, Reijo, & Clark, 2005; Leis et al., 2012; Lengerke et al., 2011; Nagata et al., 2012; Yu et al., 2011).

Bone morphogenic proteins (BMPs) are signalling molecules that are part of the TGFβ superfamily, play an important role in the regulation of cell proliferation, apoptosis and differentiation, as well as being involved in cancer

development and progression (Alarmo, Kuukasjärvi, Karhu, & Kallioniemi, 2007). In particular, BMP4 has been found to have high expression in metastatic breast cancer cell lines and is thought to have a role in the invasion and migration of breast cancer cells and also influences other migration factors such as CXCR4, a receptor that interacts with cytokine CXCL12, and this signalling axis is thought to have an important role in the homing of metastatic cancer cells to the bone (Guo, Huang, & Gong, 2012). CXCR4 has been found to be overexpressed in mammospheres as well as metastatic breast cancers (Dewan et al., 2006; Dontu et al., 2003; Guo et al., 2012; a Müller et al., 2001).

BMI1 is a polycomb group repressor, responsible for the self renewal of hematopoietic and neuronal stem cells by the repression of genes involved in senescence (Jacobs, Kieboom, Marino, Depinho, & Lohuizen, 1999). CD1d is a glycoprotein expressed on antigen presenting cells and is involved in the presentation of glycolipids to natural killer T cells (Adams & López-Sagaseta, 2011). CD1d and BMI1 have both been found to be factors involved in the self-renewal of mammary stem cells, and BMI1 has also been shown to be upregulated in the $CD44^{+}/CD24^{-}$ cell fraction of breast tumours (Dos Santos et al., 2013; S. Liu et al., 2006).

Multiple studies have suggested that markers of normal mammary stem cells, as well as components of signaling pathways involved in the maintenance of embryonic stem cells, are involved in breast cancer (Ezeh et al., 2005; Leis et al.,

2012; Lengerke et al., 2011; Nagata et al., 2012; Yu et al., 2011). These studies have shown that the expression of these markers has been related to the formation of spheres from both mammary epithelium and tumour cells and is implicated in maintaining the self-renewal of these cells, perhaps playing the same role in breast cancer development as they do with normal tissue development. Therefore, it is likely that markers of mammary stem cells and signaling molecules involved in the maintenance of embryonic stem cells may, in addition to the markers already identified through xenograft experiments, be putative markers of breast CSCs.

### 1.7 Prognostic and predictive markers for breast cancer

Breast cancer is the most common cancer among women worldwide and the second leading cause of cancer related deaths in women in North America. Due to advances in screening and prevention, the majority of breast cancers will be diagnosed at a nonmetastatic stage and be amenable to surgical removal (Cancer Care Ontario, 2011; Cardoso, Harbeck, Fallowfield, Kyriakides, & Senkus, 2012; Fisher et al., 1997; Muss et al., 2005). Currently, pathologic assessment of tumour size, grade, stage and lymph node assessment through sentinel biopsy or axillary dissection, as well as the expression of the estrogen receptor (ER), progesterone receptor (PR), and HER2 provide the most information about tumour behavior (Cheang et al., 2009; Elston & Ellis, 1991). This information, combined with age at diagnosis and patient co-morbidities,

guide clinicians in their recommendations of adjuvant hormone therapies or cytotoxic chemotherapy.

More recently, a number of predictive gene signatures have been developed to better predict survival or response to chemotherapy in breast cancer, including MammaPrint, EndoPredict, Oncotype Dx and Prosigna (Filipits et al., 2014; Soonmyung Paik et al., 2004; van't Veer et al., 2002). MammaPrint is a multigene microarray-based assay conducted using fresh or frozen tumour material that is used as a prognostic test for lymph node negative patients recently diagnosed with breast cancer (van't Veer et al., 2002). Endopredict and Oncotype Dx are designed to predict outcome of ER positive breast cancer patients specifically, and vary slightly. EndoPredict is an 11 gene qRT-PCR based assay that combines information about lymph node status and tumour size to elicit a score that assigns patients to high risk or low risk of distant recurrence (Filipits et al., 2011). This score adds information to traditional pathological variables and has been shown to significantly alter treatment plans such that, in one study, 25% of patients opted to forgo planned chemotherapy (Müller et al., 2013). Prosigna (PAM50) recently received FDA approval for use in women with early stage hormone receptor negative or positive and/or 1 – 3 node positive breast cancer, and utilises RNA extracted from FFPE samples on the NanoString nCounter platform (Cuzick et al., 2011; Nielsen et al., 2014). The assay provides a risk category of low, medium or high and a numerical score for the risk of distant

metastases at 10 years for post-menopausal women (Filipits et al., 2011, 2014). It also provides a tumour subtype: luminal A, luminal B, HER2 enriched, basal-like or normal-like (Filipits et al., 2014).

### 1.8 CSCs as biomarkers in breast cancer

There are a number of groups that have shown that cancer stem cells can be biomarkers for patient survival and that these markers correlate with aggressive tumour characteristics in breast cancer. Analysis of ALDH1 and $CD44^{+}/CD24^{low}$ expression by immunohistochemistry (IHC) has been shown to be positively correlated with high tumour grade and a high mitotic index, but expression of these markers is not able to predict survival in familial breast tumours (A. Bane et al., 2013). Similar observations have been reported in invasive ductal carcinomas, where $CD44^{+}/CD24^{low}$ and ALDH1 expression was found to correlate with ER negativity and expression of CD133 was more common in younger patients and triple negative (ER, PR, and HER2 negative) tumours, although they were also unable to show a correlation with survival (Currie et al., 2013). The expression of CD44/CD24 and ALDH to identify breast CSC fraction has previously been performed using flow cytometry. While this may be transferrable to IHC for evaluating CD44 and CD24, since both methods are assessed using cell surface markers, the ALDEFLUOR assay is a measure of ALDH enzymatic activity, which is not captured using IHC. A number of studies have been performed using

immunohistochemical markers for ALDH1 (Eirew et al., 2012; Ginestier et al., 2007; Marcato, Dean, Pan, & Araslanova, 2011; Ricardo et al., 2011). Interestingly, while the original report identifying ALDH activity as a marker of CSCs utilised an antibody against the ALDH1A1 isoform (Ginestier et al., 2007) a study by Marcato et al. (2011) suggests that the different isoforms of ALDH1 correlate differently with respect to cancer metastasis. Immunolohistochemical studies using the isoform ALDH1A1 revealed that expression of this marker is not correlated with breast cancer metastasis and instead it has been suggested that a more robust marker is the isoform ALDH1A3, and its expression has been shown to be significantly associated with tumour grade, metastasis, and cancer stage in formalin fixed patient samples (Marcato et al., 2011). Additionally, ALDH1A3 has been shown to be the isoform most highly enriched in luminal progenitor cells (identified by $CD49f^{+}EpCAM^{+}CD133^{+}$ expressing cells) from normal mammary epithelium (Eirew et al., 2012).

Gene signatures developed from microarray expression data of CSCs have also been used as biomarkers in breast cancer (Glinsky, Berezovska, & Glinskii, 2005; Lahad, Mills, & Coombes, 2005). Gene expression data from $CD44^{+}/CD24^{low/-}$ tumour cells have been compared to normal breast epithelium to develop CSC signatures, which are able to predict distant metastasis free survival in independent patient datasets (Liu et al., 2007; Shipitsin et al., 2007). Similarly, expression data from ALDH (ALDEFUOR) positive cells has also been used to

develop a CSC signature which separated patients into CSC-like and non-CSC-like groups; CSC-like patient tumours are more likely to be high grade, triple negative and metastasize to the lymph node, as well as have poorer metastasis free survival (Charafe-Jauffret et al., 2013).

Xenograft formation in mice has also been shown to be a biomarker for breast cancer. Patient tumours that are able to form tumours in mice have been shown to possess more aggressive characteristics, typically high tumour grade and ER negativity, and successful engraftment has also been shown to correlate with poor patient survival (DeRose et al., 2011; Petrillo et al., 2012; Zhang et al., 2013).

### 1.9 Molecular subtypes of breast cancer and prognosis

Breast cancers have been found to have varied prognosis based on expression of a variety of molecular markers contributing to breast cancer heterogeneity. Gene expression analysis has been able to divide invasive breast tumours into different molecular subtypes, which have distinct clinical outcomes (Sørlie et al., 2001). Perou et al. (2000) performed gene expression profiling on approximately 100 invasive breast cancers and, using a hierarchical clustering algorithm that grouped genes based on the similarity of their expression patterns, they were able to characterize invasive tumour samples. They found that invasive breast cancers can be broadly classified into two main groups: ER positive,

characterized by the high expression of genes in normal luminal epithelial cells of the breast, and ER negative (Perou et al., 2000). Further research on luminal/ER positive tumours allowed researchers to further categorize them into subgroups: Luminal A (characterised by expression of ER and/or PR (progesterone receptor), with a low Ki67 (a marker for proliferation) index by IHC and luminal B (characterised by ER and/or PR expression as well as high HER2 and/or Ki67 expression). ER negative tumours were categorized into basal-like (triple-negative for ER, PR and HER2 but positive for CK5 and/or EGFR), human epidermal growth factor receptor 2 (HER2) overexpressing and normal breast-like subgroups (Sørlie et al., 2001). These subgroups were correlated with different clinical outcomes, with Luminal A tumours having the best outcomes, luminal B tumours having intermediate outcomes and basal-like and HER2+ tumours having the worst outcomes (Sorlie et al., 2003).

More recently, other subtypes of breast cancer have been identified and added to this molecular taxonomy; including molecular apocrine and 'Claudin-low' (Farmer et al., 2005; Herschkowitz et al., 2007). The claudin-low subtype was identified by gene expression analysis (Herschkowitz et al., 2007). This subtype is typically ER, PR and HER2 negative (triple negative) and characterised by low expression of claudins 3, 4 and 7 and E-cadherin (Herschkowitz et al., 2007). It is further characterized by high expression of mesenchymal and immune response genes and is associated with a poor prognosis (Prat et al., 2010).

Claudin-low tumours are thought to have stem-cell features because they are significantly enriched for a tumour-initiating cell signature identified by isolating the $CD44^{+}CD24^{-}$ fraction of breast epithelial cells and comparing their gene expression with that of other non $CD44^{+}/CD24^{-}$ cell fractions (Creighton et al., 2009). The signature is highly enriched for mesenchymal and epithelial to mesenchymal (EMT) features and was significantly correlated with the claudin-low subtype which suggests that this subtype is naturally enriched for CSCs (Creighton et al., 2009). EMT is a shift in cell phenotype and plays a key role in development, where epithelial cells break down contact between neighbouring cells and extracellular matrix, and migrate to other locations, a process which is thought to contribute to metastatic tumour growth and is considered one of the characteristic features of stem cells (Mani et al., 2008). Inducing EMT through expression of Snail or Twist transcription factors in non-tumourigenic human mammary epithelial cells resulted in most mesenchymal cells acquiring a $CD44^{high}/CD24^{low}$ phenotype, and these cells displayed stem cell-like characteristics including the ability to form mammospheres, as well as the ability to differentiate into cells expressing both luminal and myoepithelial markers (Mani et al., 2008)

The diversity of breast cancer molecular subtypes and the evolution of our understanding of the normal stem and progenitor cell populations of the human breast have given rise to a theory to account for the origins of these different

subtypes of breast cancer (Lim et al., 2009; Prat & Perou, 2009). A tumour could arise either from a transformed stem/progenitor cell and give rise to a heterogeneous population of cells, or a cell in an already heterogeneous tumour could acquire the capacity for self-renewal. In both of these possibilities the majority of the tumour cells are differentiated and have limited proliferative capabilities whereas the small CSC population maintains the tumour (Prat & Perou, 2009). The molecular heterogeneity of breast tumours is hypothesized to result from each subtype arising from a different cell within the mammary epithelial cell hierarchy. The subtypes with poorer prognoses, like claudin-low and basal-like tumours, have expression profiles similar to the mammary stem cell or luminal progenitor populations respectively, and subtypes with more favourable prognoses like Luminal A and Luminal B subtypes have profiles that are similar to more differentiated luminal cells (supplementary Figure 4) (Lim et al., 2009). This suggests that each molecular subtype could arise from independent pools of CSCs, which may be identified by unique markers for each breast cancer subtype. There has been some evidence that these differences can be used to reliably separate tumours into good prognosis and poor prognosis groups by using gene expression signatures for CSCs (Glinsky et al., 2005; Lahad et al., 2005; R. Liu et al., 2007; Shipitsin et al., 2007).

## STUDY OBJECTIVES

The work to date has succeeded in confirming the existence of breast CSCs in a very small number of predominantly metastatic breast cancers, enriching for them with antibodies to cell surface markers or endogenous enzymes and demonstrating their unique tumourigenic capacity. However, the clinical significance of breast CSCs is unclear, and the connection between the various subtypes of breast cancer and CSC frequency hasn't been fully investigated. Hence, whether the frequency of CSCs in *primary* human breast tumours is associated with poor prognosis is uncertain. Fundamental questions that remain to be addressed include:

- Whether the *quantity* of CSCs/TICs in a primary tumour is related to aggressive tumour characteristics and/or behaviour.
- What are the most appropriate markers to *identify* the breast CSC/TICs population for the purposes of patient management and treatment

The objective of this work is to better understand the relationship between breast CSCs and patient outcome by:

1. Examining the frequency of CSCs in primary human cancers through limiting dilution assays and sphere forming assays
2. Correlating the presence and frequency of CSCs in primary human cancers with pathologic tumour characteristics and prognosis

3. Identifying markers of CSC that may be of prognostic or predictive utility in the treatment of breast cancer patients.

# METHODS

## 2.1 Xenografts and LDCT assays

### *2.1.1 Obtaining fresh primary human breast tumours*

Fresh tumour samples were collected prospectively at surgical resection from St. Joseph's hospital and Juravinski hospital (Hamilton, ON) with the collaboration of Dr. Peter Lovrics and Dr. Nicole Hodgson under approval from the McMaster research ethics board (REB: #09-185) and after obtaining informed consent from patients. The fresh tumour samples were transported in HBSS (Hank's Balanced Salt Solution), frozen in freezing media (90% FBS, 10% DMSO) and stored in liquid nitrogen. Depending on the size of tissue available, tumour cells were isolated for xenograft injections, sphere forming assays or both.

### *2.1.2 Tumour cell isolation*

Tissue was minced using sterile scissors and scalpels and added to Versene (3mL/g of tumour). RPMI media (2% FBS) containing collagenase and trypsin (7mL/g of tumour) was added to the tumour and Versene mixture and incubated for 15 minutes. After incubation, the solution was pipetted 15-20 times and incubated again for 15 minutes. This mixture was then mixed with 10mL RPMI media (2%FBS) and passed through a 40μm cell sieve and spun at 1500rpm for 15

minutes. The pellet was resuspended in F12 media and live cell number was counted (using trypan blue exclusion) and spun down for 5 minutes (Kondratyev et al., 2012; Kurpios et al., 2009). Approximately $10^5$ cells were mixed with 25μL (per injection site) of a 1:1 ratio of Matrigel and PBS containing 5% FBS and mixed with fibroblasts for injection into mice.

### *2.1.3 Culture of human reduction mammary fibroblasts (RMFs)*

RMFs were obtained from the Kuperwasser lab and grown in DMEM (10%FBS). Fibroblasts are irradiated 24 hours before injection and are mixed in equal parts with normal fibroblasts, in a 1:1 ratio of Matrigel and PBS containing 5% FBS. 25μL was mixed with the tumour cells in matrigel for injection into mice for humanization or co-injection with tumour cells (Proia & Kuperwasser, 2006).

### *2.1.4 Establishing xenografts from primary tissue*

For the establishment of mouse xenografts and LDCT assays, tumour cells were isolated (as described above) and approximately $10^5$ live cells were injected orthotopically into the cleared, humanized mammary fat pad #4 of NOD/SCID IL2R$\gamma^{null}$ mice as described previously (Proia and Kuperwasser, 2006). The mice were observed weekly and resulting xenografts were harvested from sacrificed mice when they reached a certain size (as determined by the Animal Utilisation Protocol, AUP#100104) and tumour initiating cell frequencies are calculated from

the LCDT assays using the Extreme Limiting Dilution Analysis (ELDA) web-based software (Hu & Smyth, 2009).

### *2.1.5 Patient-derived xenograft propagation*

Cells were dissociated from sections of primary xenografts as described above. $10^5$ live cells were mixed with 50μL of a 1:1 ratio of Matrigel and PBS containing 5% FBS for subcutaneous injection into mice.

### *2.1.6 Histologic evaluation of xenografts and primary tumours*

Xenograft tissue was fixed in formalin for 24 hours and embedded in paraffin. To ensure the xenografts established were of human origin, sections were stained with an antibody against human mitochondria (Antibodies online, Cat# ABIN361348). Immunohistochemistry was performed as previously described (Bane et al., 2013). Briefly, 4μM sections were cut and stained for ER, PR HER2, CK5, EGFR, and Ki67. Antigen retrieval was carried out in a Micromed T/T Mega Microwave Processing Lab Station (ESBE Scientific, Markham, Ontario, Canada) and sections were developed with diaminobenzidine tetrahydrochloride (DAB) and counterstained in Mayer's hematoxylin.

## 2.2 Spheres and sphere forming assay

### *2.2.1 Sphere forming assays*

Sphere forming assays were performed by plating tumour cells isolated from primary human breast tumours (as described above) in human stem cell media, which comprises DMEM: Ham's F-12 (3:1), 4 µg/ml of B-27, 20 ng/ml EGF, 40 ng/ml FGF-2 and 4 ng/ml Heparin (Invitrogen, Carlsbad, CA, USA) (Kurpios et al., 2009), at a density of 30,000 cells/ml in an ultra low attachment 24 well plate (in triplicate). Cells were gown as previously described to yield tumourspheres (Reynolds and Weiss, 1992). The number of tumourspheres was counted after 7 days, and the sphere forming frequency (SFF) of each tumour was determined (number of spheres/number of live primary tumour cells seeded). Secondary sphere forming assays were conducted by obtaining spheres from the primary assay and triturating them with TEDTA at 37°C to dissociate spheres into single cells. Cells were counted using the trypan blue exclusion assay to assess cell viability, and viable tumour cells were replated in fresh human stem cell media. Secondary spheres were counted after 7 days and the secondary SFF was determined (number of spheres/number of live tumour cells seeded).

### *2.2.2 Tissue culture of adherent cells and spheres*

10 human breast cancer cell lines representative of the different molecular subtypes were grown in serum containing media as indicated (Supplementary table 1). One T75 flask of adherent cells (approximately $10^6$ cells) was pelleted and stored in lysis buffer for RNA extraction. Spheres were converted from adherent cells by plating at a density of 30,000 cells/ml in human stem cell media for four days. The resulting spheres were dissociated, replated and allowed to grow to achieve a homogenous sphere culture. Spheres were pelleted and stored in lysis buffer for RNA extraction.

## 2.3 Chart review and statistical analysis

### *2.3.1 Chart Review*

Charts were obtained, after REB approval, from patients who submitted fresh tumour samples for the CSC study. Pathological characteristics and recurrence and survival information were compared with CSC frequency measured by sphere forming assays. Pathology reports for each patient provided data about tumour characteristics including: tumour size, tumour grade, lymph node status, lympho-vascular invasion and ER, PR and HER2 status determined by IHC, or fluorescence in situ hybridization (FISH) if IHC was indeterminate (2+) for

HER2. Case notes were used to identify therapies employed, as well as recurrence and survival status.

### *2.3.2 Statistical tests*

Statistical analyses were performed with the collaboration of a clinical statistician, Dr. Gregory Pond. Xenograft and sphere formation was compared to patient outcome and pathological characteristics. Fisher's exact tests (for binary variables), $\chi^2$ tests (for categorical variables), Wilcox rank sum tests (for continuous variables) and log-rank tests (for survival analyses) were used to determine if there were significant differences between patients with or without sphere forming or xenograft forming ability.

## 2.4 RNA analysis of CSC markers

### *2.4.1 NanoString*

The NanoString nCounter system was used to measure the RNA expression of candidate CSC markers (Supplementary table 2) in breast cancer cell lines (adherent cells and spheres). RNA was extracted from cell lysate with the Qiagen assay kit using the manufacturer's protocol. RNA was analysed using the Agilent bioanalyser and approximately 200ng of RNA was used for the NanoString assay. NanoString constructed a custom codeset using the candidate CSC markers, and the samples were run at the Farncombe Metagenomic Facility (McMaster

University, Hamilton, ON) according to recommendations outlined by NanoString Technologies regarding mRNA sample preparation, hybridization, detection and scanning, and data normalization (Geiss et al., 2008).

### *2.4.2 NanoString data analysis*

NanoString expression counts were normalized to the positive control and the housekeeping genes using the geometric mean by the nSolver software from NanoString technologies. This normalized data was then used to plot expression counts and to calculate fold change between spheres and adherent cells for each cell line using the following formula: (expression value for adherent cells/expression value for spheres). Cell lines were grouped by subtype (luminal or basal) and the 95% confidence interval (CI95) of the fold change ratios was calculated for each gene in each subtype to determine which genes had expression that was significantly higher in spheres as compared with adherent cells.

### *2.4.3 CSC genes in microarray analysis*

Publicly available gene expression databases using in silico microarray data on the HG-U133A Affymetrix GeneChip were used to test the expression of the CSC genes in a large cohort of 1, 593 patient breast tumours. A centroid for each subtype was calculated based in the expression of the CSC genes. Tumours were assigned a subtype based on their correlation to the centroid.

## 2.5 IHC of Breast tumours

### *2.5.1 Accelerated Hypofractionated Whole Breast Irradiation (AHWBI) Trial Samples*

As part of a clinical trial examining the effects of two different schedules of whole breast irradiation following breast conserving surgery, 1,234 stage 1 or stage 2, lymph node negative, primary invasive breast cancers had one representative formalin fixed paraffin embedded (FFPE) tumour block available for pathology review and tissue microarray (TMA) construction. Ten year local recurrence (LR) and overall survival (OS) was available for all study participants (Bane et al., 2014).

### *2.5.2 Tissue Microarray Construction*

A hematoxylin and eosin (H&E) stained section of each tumor block received was prepared to confirm the diagnosis and circle the area of invasive tumour with permanent ink for tissue microarray (TMA) construction. Three 0.6mm cores of tissue were taken from the paraffin tumour block and used for TMA construction (Pathology Device, Sun Praire, WI) as previously described (Bane et al., 2007).

### *2.5.3 Immunohistochemistry*

4 Sections of the TMA were cut and stained for the IHC markers described above, as well as CD24, CD44, ALDH1 and CD49f. An Allred score (Allred, Harvey,

Berardo, & Clark, 1998) was used to score each marker. The Allred score is a semi- quantitative analysis that accounts for the percentage of cells stained (1=<1%, 2=1-10%, 3=11-33%, 4=34-66%, 5=67-100%) and the intensity of staining (1 = weak, 2 = moderate, 3 = strong). Previously validated cut-offs for ER and PR were used (0, 2=negative,3- 8=positive) (Harvey, Clark, Osborne, & Allred, 1999; Mohsin et al., 2004). HER2 was assessed by strong complete membranous staining with cut-off of > 5 to indicate positivity (Wolff et al., 2007). For CK5, EGFR, CD44, CD24, CD49f and ALDH1 a score of ≥ 5 was considered positive.

# RESULTS

## *3.1 Xenografts and LCDT assays from primary human breast tumour samples*

We received 136 tumours from consenting patients, which we isolated and injected into the number 4 mammary fat pad of humanized NSG mice. An injection was considered successful if the mice were viable for at least 8 months after the procedure (i.e. the mice did not die of an infection immediately after the procedure, or had to be euthanized due to other sickness). 77 injections were considered successful and 8 of 77 (10.39%) primary human tumours successfully yielded xenografts when transplanted under the conditions described (Table 1).

To determine the molecular subtype of the xenografts and their parent tumours we used protein expression of a limited panel of immunohistochemical markers (ER, PR, HER2, CK5, EGFR and Ki67) as described by Nielsen and colleagues (Nielsen et al., 2004) to determine an approximate tumour subtype. In all instances the xenograft faithfully matched the morphology (Figure 1) and molecular subtype of the parent tumour (Figure 2, A – G). Furthermore, the 7 generated xenografts were representative of multiple molecular subtypes, including three luminal B tumours, two HER2 overexpressing tumours and two triple negative tumours (Table 1). The primary xenografts that formed varied in size as well as latency. Tumour latencies shortened with subsequent xenograft passages (Table 1).

LDCT assays were conducted using tissue from the primary xenografts to determine the tumour initiating cell frequency in these tumours. From the tumours that formed successful xenografts, we were able to isolate a sufficient number of cells from seven out of eight xenografts to be able to inject varying numbers of cells as part of the LDCT assay (supplementary table 3). A tumour initiating cell frequency was subsequently determined from the tumours that formed using computer software (ELDA) that incorporates the number of cells injected and the time to tumour formation (Table 2). In five tumours a TIC frequency could be generated, and the TIC frequency ranged from 1:166,010 to 1:329. Due to low numbers and hence inadequate statistical power we were unable to determine whether the TIC frequency correlated with any pathological or clinical variables in our patient group.

Since the results obtained from the LDCT assays were not sufficient for a robust analysis of CSC frequency as it relates to tumour pathology and patient outcome, we used the tumour engraftment and successful xenograft formation as a surrogate analysis. To determine whether xenograft formation is correlated with any known clinical or pathological tumour characteristics, we compared those tumours capable of engrafting with those tumours that did not yield xenografts for characteristics such as tumour size, grade, stage and receptor status. The ability of a primary breast tumour to engraft was found to be significantly associated with high tumour grade ($p=0.009$). Although not statistically significant, we also

observed a trend towards molecular subtype and ER negativity (p=0.093 and p=0.098 respectively) as well as PR negativity (p=0.070) being related to engraftment (Table 3). There was no significant association between the ability of a tumour to engraft and patients' overall survival (p=0.7). Interestingly, 25% of patients whose tumours formed a xenografts developed distant metastases compared to 9% of patients whose tumours did not form xenografts, although this relationship was not statistically significant it might be considered clinically significant (p=0.21).

### *3.2 Sphere forming assays*

From the 136 patient tumours received, sphere forming assays were performed on cells isolated from 68 primary tumour samples (supplementary table 4). With the number of cells plated, 49 (72%) of these tumors were able to form spheres in serum free media. We were then able to calculate the sphere-forming frequency of the tumour based on the number of spheres formed and the number of cells seeded. Of the tumours that were able to form spheres, 9 were also able to form secondary spheres when the spheres were dissociated and replated, demonstrating the sphere forming cell's ability to self renew. We examined the ability of tumours to form primary or secondary spheres when compared to clinical outcome and pathological tumour parameters.

The ability of a tumour to form a sphere in the primary sphere forming assay had no statistically significant relationships with tumour parameters such as tumour grade, presence or absence of lymphovascular invasion, tumour stage, hormone receptor or HER2 status or molecular subtype (Table 4). However, there was a trend between primary sphere forming efficiency and a younger age at diagnosis ($p=0.096$), and higher-grade tumours ($p=0.19$). No correlation with patient survival and primary sphere forming ability could be demonstrated.

Sphere forming frequency (SFF) was also compared to clinicopathological parameters to determine whether the frequency of spheres formed was a prognostic factor. There were no statistically significant relationships between SFF and tumour characteristics such as tumour grade, presence or absence of lymphovascular invasion, tumour stage, hormone receptor or HER2 status or molecular subtype (Table 4). There was also a trend with SFF and younger age at diagnosis ($p=0.070$). We were also unable to demonstrate a relationship between primary SFF and survival.

When these spheres were dissociated and replated in serum free media, the secondary sphere forming ability was compared to pathological tumour characteristics. The comparison suggests that there is a trend (nonsignificant) towards larger tumours having an increased ability to form secondary spheres ($p=0.08$, Table 5).

### *3.3 Expression of potential CSC markers in human breast cancer cell lines*

Potential CSC markers were identified from the literature (supplementary table 2) and a custom codeset was synthesized by NanoString to be used in the NanoString nCounter system. The codeset was run on RNA extracted from human breast cancer cell lines representative of different breast cancer subtypes, grown adherently and as spheres, to compare differences in RNA expression between these growth conditions. RNA counts were normalized to the housekeeping genes and positive and negative controls using the nSolver program from NanoString technologies. Expression values had a wide range depending on the gene examined, and gene expression varied between cell lines. The clinical markers ER, PR and HER2 were examined in each of the cell lines, and luminal cell lines (both grown adherently and as spheres) were found to have high ER/PR expression, whereas spheres and adherent cells from basal cell lines had absent or low expression of ER/PR (Figure 3).

When performing cluster analyses on this data, the genes we used were able to cluster the cell lines based on molecular subtype, i.e. Luminal, Basal A and Basal B (Figure 4). We compared the differences in expression of the CSC genes between spheres and adherent cells for each cell line by calculating the fold-change ratio of expression of each gene between adherent cells and spheres. Cell lines were grouped into luminal and basal subtypes and a 95% confidence interval (CI95) of these ratios was calculated for each gene, in each subtype. If

the CI95 was greater than 1, these genes were considered to have significantly increased expression in adherent cells vs spheres, and if the CI95 was less than 1, these genes were considered to have significantly higher expression in spheres vs adherent cells. As per this analysis, EpCAM and CD49f and KLF4 had significantly higher expression in spheres vs. adherent cells in both the luminal and basal subtypes (Figures 5 and 6). BMP4 had significantly higher expression in spheres compared to adherent cells in the basal subtype (Figure 5), and CD24, CD44, ECAD, CXCR4 and HER2 had significantly higher expression in spheres in the luminal subtype (Figure 6). None of the genes tested had significantly higher expression in adherent cells compared to spheres in either the luminal or the basal subtype.

Since these genes were able to cluster the cell lines based on subtype, we tested the genes together as a signature for its ability to classify patient tumours into the different molecular subtypes in a large cohort of primary breast tumours using publicly available gene expression microarray data. The gene signature was able to broadly cluster primary tumours into different molecular previously described subtypes (Figure 7). The classification of the tumours into the different subtypes revealed differences in overall survival and disease-free survival according to subtype (Figure 8 A and B), where basal and HER2 tumours have the worst prognosis and luminal A tumours having the best prognosis.

### *3.4 Expression of CSC markers in breast tumours by IHC*

Immunohistochemical expression of CD44, CD24, CD49f and ALDH1 was evaluated in FFPE samples of 1,234 patient breast tumours (supplementary figure 5), and an Allred score was assigned in 904 (73.3%) of these tumours. An Allred score cut-off of ≥ 5 was to indicate positive expression. IHC expression of these CSC markers was varied in our tumour cohort, with 8.9% of the tumours expressing ALDH1, 70.6% expressing CD44, 47% expressing CD24, and 24.9% expressing CD49f (Table 6).

The expression of these markers was compared to tumour pathological characteristics and patient outcome. The expression of each of the four markers was significantly associated with molecular subtype ($p<0.001$) (Table 6). In addition, expression of ALDH1 and CD49f was significantly correlated with age younger than 50 years at diagnosis ($p=0.001$, $p<0.001$ respectively), tumour size larger than 2cm ($p<0.001$, $p=0.015$ respectively), and high grade ($p<0.001$). CD24 expression was significantly correlated with age at diagnosis ($p=0.017$), and tumour size larger than 2cm ($p=0.006$) (Table 6). Expression of ALDH1, CD44 and CD49f were not correlated with patient survival, however expression of CD24 was correlated with disease-free survival ($p=0.008$, Table 7).

# DISCUSSION

## *4.1 Xenografts of primary human breast tumour samples*

From the successful injection of 77 primary tumour samples, 8 patient derived tumour xenografts were established in mice. The primary xenografts were found to closely resemble the patient tumour both in terms of tumour morphology and IHC marker expression, which was subsequently used to determine the molecular subtype of the tumour.

Since the amount of tissue we received from patient samples was inadequate to perform limiting dilution cell transplant assays, the primary xenografts were used to perform LDCT assays to assess functional tumour initiating cell frequency. While it was our intention to compare the tumour initiating cell frequency as determined by limiting dilution assays to patient survival and tumour characteristics, the limited number of tumours we were able to perform these assays with were not sufficient to perform robust comparisons. Nonetheless, it is interesting that the tumour with the highest tumour initiating cell frequency (1:329 #43, Table 2) was a large 8cm tumour, which was triple negative and the only sample (from the eight that formed xenografts) that was derived post neoadjuvant chemotherapy. Traditional therapies, including those used for neoadjuvant treatment, have been shown to target the more differentiated cells in a tumour and reduce tumour bulk, but the tumour initiating frequency of

cells is thought to be more resistant to these therapies (Creighton et al., 2009). Others have shown that tumours remaining post neo-adjuvant chemotherapy are particularly enriched for TICs with enhanced engraftment ability (Li et al., 2008; Zhang et al., 2013). Unfortunately, this patient (#43) went on to develop distant metastases in the lung in a very short amount of time (approximately 18 months) and succumbed to her disease.

Since we were not able to assess the quantity of TICs in relation to clinical tumour parameters, we used xenograft formation as a surrogate marker for the presence of TICs. All of the xenografts that were formed resulted from high (grade III) grade tumours, demonstrating that the more aggressive tumours have better engraftment rates. In addition, the ER status of the tumour and molecular subtype were also factors important for tissue engraftment. These findings have been demonstrated by other studies, namely the tendency for triple negative xenografts to engraft more readily than other subtypes, as well as attaining more stable engraftment of grade III tumours than grade I or grade II tumours (Petrillo et al., 2012; Zhang et al., 2013). Interestingly, while we were able to engraft luminal B, HER2 positive and triple negative tumours, despite injecting many primary luminal A tumour samples (19 samples) we were unable to obtain a xenograft. The limited ability to successfully engraft luminal A tumours has also been reported by other groups (Marangoni et al., 2007; Petrillo et al., 2012; Zhang et al., 2013). It has been suggested that the high proliferation of triple negative

tumours is a reason for the superior engraftment of these tumours compared to luminal A tumours (Petrillo et al., 2012). Additionally, luminal A tumours are one of the least aggressive subtypes of breast cancer (Sorlie et al., 2003) and, according to the CSC model, they may originate from more differentiated luminal cells when compared to the triple negative subtype (Supplementary figure 4; Prat & Perou, 2009). It is possible that since luminal A tumours arise from more differentiated cells, they possess fewer TICs than other tumour subtypes, or different TICs that are not amenable to the engraftment process.

There was a trend (nonsignificant) between the ability of a tumour to form a xenograft and disease free survival ($p=0.21$) (Table 2). The average follow up time for these patients was three years, and overall survival during this time was high (88.4%) with only 8 events (Supplementary table 5). While the correlation between the ability of a tumour to form a xenograft and disease-free survival is not statistically significant, this relationship is clinically significant since 25% of the patients whose tumours formed xenografts developed distant metastases. The lack of significance with this comparison may simply be a time dependent factor, and with additional clinical follow-up or larger numbers of patients it is possible that this trend may become significant. Although it is not conclusively shown that a primary tumour's ability to xenograft is related directly to patient outcome, engraftment is correlated with aggressive pathological tumour characteristics, which are known to contribute to poor patient survival.

Thus far, the majority of studies, in breast and other solid tumours, have performed limiting dilution assays using cells sorted for potential CSC makers to highlight the increased tumour initiating ability of cells expressing these marker phenotypes (e.g. ALDEFLUOR+, $CD44^{high}/CD24^{low}$, CD133+) compared to cells that do not have this marker expression (Al-Hajj et al., 2003; Charafe-Jauffret et al., 2013; Shmelkov et al., 2008; Singh, Hawkins, Clarke, & Squire, 2004). In contrast, our study was performed with no pre-sorting of cells based on marker expression in an attempt to elucidate the raw TIC frequency within a tumour, potentially capturing cells that may be missed in the sorting process. However, the technical difficulty, time, expense and the requirement for fresh tissue are barriers to the adoption of this assay as a clinical tool.

### *4.2 Sphere-forming assays*

The relationship between sphere formation from patient tumours and patient survival has not been extensively explored previously in breast cancer. While there have been reports regarding brain tumours that suggest that the most aggressive medulloblastomas have the highest secondary sphere forming ability, these were established from cells that were previously sorted for CD133, (Singh et al., 2004; Singh et al., 2003). Our study is unique in that the cell population was not previously sorted for any markers before being tested for sphere forming ability.

In this study, we were unable to demonstrate a relationship between the ability of a tumour to form a sphere with any tumour pathological variable. There was a trend between sphere formation and higher-grade tumours and a younger age at diagnosis, features that are typically associated with more aggressive tumours. Patients with a younger age at diagnosis also trended towards higher sphere-forming frequencies. However, we were unable to show a correlation between either a tumours ability to form a sphere or sphere forming frequency and patient survival. This is possibly due to the short follow up time of 3 years with only 5 distant metastasis events observed in patients whose tumours formed a sphere compared with 3 events in patients whose tumours did not form a sphere. Secondary sphere formation was not significantly correlated with any tumour pathological variables, although there was a trend towards larger tumours and an ability to form secondary spheres.

For a small number of tumours, we had an estimate of the frequency of cells that formed tumours or spheres from both the LDCT and sphere forming assays and, in general, the sphere forming frequency obtained from the SFAs was significantly higher. Since LDCT assays are considered the gold standard assay for quantifying the number of tumour initiating cells present in the tumour, they are likely a more accurate representation of functional tumour initiating cell frequency compared to sphere forming assays (Visvader & Lindeman, 2008). This leads to the conclusion that the sphere-forming frequency of a tumour shows

little correlation with known adverse tumour pathologic characteristics or patient outcome although our numbers are small. Secondarily it appears that sphere forming assays may overestimate the CSC/TIC frequency of a tumour when sphere forming frequencies are compared to functional TICs from LCDT assays.

### *4.3 Expression of potential CSC markers in human breast cancer cell lines*

The data from our sphere forming analyses of patient tumour samples suggest that it is not a feasible measure of stem cell frequency, at least in the clinical setting. We attempted to produce enough spheres from assays of patient tumours to be able to test potential CSC markers expression of these cells, however the small number of spheres that were formed with the limited amount of tumour tissue available were not sufficient to be able to paraffin embed the spheres and robustly test for markers, either through IHC, or gene expression since there were not a sufficient number of cells from which we would be able to extract RNA.

The majority of CSCs have been identified through cell surface markers from cell sorting experiments, however the gene expression of some of these genes has been explored through methods like RT-PCR, albeit from cells that were previously sorted in most cases. However, it is unlikely that cell sorting experiments will be practical in a clinical setting due to the limited amount of tissue samples available. While immunohistochemistry is commonly used and

well established to test for protein expression in the clinic, especially in the case of ER PR and HER2 which are known prognostic markers, there has been a movement towards gene expression based clinical tests to predict patient survival and response to therapy especially with the development of tests such as Oncotype Dx, Mammaprint, and Endopredict (Albain et al., 2010; Filipits et al., 2011; Paik et al., 2004; van't Veer et al., 2002). With their approval for clinical utility in patients, clinicians have more information about predicted patient survival and are better equipped to make decisions about treatment regimens (Blohmer et al., 2013; Müller et al., 2013; Saghatchian et al., 2013). The success of these tests suggests that the identification of markers based on RNA expression will be more useful clinically, especially with the consideration that CSCs are likely to express a number of different markers that may not be easily captured through IHC.

Therefore, we sought to identify the expression of potential CSC markers compiled from the literature (Supplementary table 2) by exploring their gene expression in adherent cells and spheres from human breast tumour cell lines, with the objective of validating some of these markers on a cohort of patient tumour samples. The rationale for these experiments was that sphere cultures are enriched for CSCs compared to adherently grown cells and that they would be expected to express genes that are related to stemness at a higher level that their adherently grown counterparts.

Our CSC codeset included the clinical markers ER, PR, HER2 and Ki67. These markers had the expected expression pattern of ER, PR and HER2 based on their classification as luminal, basal A or basal B from previous studies (Neve et al., 2006). Differential gene expression between adherent cells and spheres for each cell line was represented as fold-change ratios, and the 95% confidence interval for each gene for the basal and luminal subtypes was used to determine genes which had higher expression in spheres or adherent cells in each subtype. Interestingly, the only genes that were expressed at a significantly higher level in spheres compared to adherent cells in both the basal and luminal cell line subtypes, were EpCAM, CD49f and KLF4. The luminal subtype had a number of genes the were more highly expressed in spheres, including CD44, CD24, CXCR4 E-cadherin and HER2, whereas spheres in the basal subtype had significantly higher expression of BMP4.

There have been reports that propose that the different subtypes of breast cancer may have different cells of origin, suggesting that there are different pools of CSCs that might give rise to a tumour and that these cells may be identified through different markers or combinations of markers (Lim et al., 2009; Prat & Perou, 2009). Studies on CSCs, which focused on functional tumour initiation properties of these cells through xenograft formation, found that there were populations of cells identified by sorting for CD133+ and $CD44^{+}/CD24^{-}$ that were

capable of forming tumours with few cells injected, but that these were distinct cell populations with little overlap (Wright et al., 2008).

When we used our expression data to perform clustering based on these genes, they were able to separate the cell lines we tested based on their molecular subtype. This is an interesting finding especially with the development of Prosigna, a clinically approved test that separates patient tumours into molecular subtypes based on the expression of 50 genes and is able to predict relapse based on the expression of these genes (Parker et al., 2009). When we tested these genes as a signature on patient samples using data from a microarray platform, they were able to differentiate between the different molecular subtypes in primary tumour samples.

Since the results of the NanoString analyses revealed different genes which seemed to be important for sphere formation in different molecular subtypes, we examined whether these gene were prognostic in the different subtypes of breast cancer in a large cohort of breast tumours. The signature as a whole was able to classify tumours into subtypes, and these subtypes had differences in patient survival, similar to what has been shown previously (Perou et al., 2000; Sørlie et al., 2001). This suggests that the genes are prognostic for patient outcome in the different molecular subtypes when used as a signature, although whether individual genes are prognostic, or whether a subset of these

genes are prognostic within certain breast cancer subtypes will require further analysis.

### *4.4 Expression of CSC markers in breast tumours by IHC*

In addition to the Nanostring and in silico work, we also looked at the expression of ALDH1, CD44, CD24 and CD49f by IHC in a large number of patient tumours accrued from the AHWBI trial. Expression of each of the markers was significantly correlated with molecular subtype, and a subset of them were also correlated with a number of aggressive tumour characteristics including larger tumour size and high tumour grade, as well as a younger age at diagnosis. The correlation between these markers and molecular subtypes indicates that there are different levels of expression in each subtype, suggesting that each marker may be prognostic in a subtype specific manner.

Although ALDH1, CD44 and CD49f expression was associated with a number of tumour features that are associated with aggressiveness, only CD24 expression was correlated with poorer disease-free survival (HR=1.45, p=0.008). This is of particular interest since breast CSCs have traditionally been characterized by low CD24 expression (in conjunction with high expression of CD44), however expression of CD24 has also been shown to be a marker of aggressive breast tumours (Kristiansen et al., 2003).

Thus far, the expression of $CD44^{+}/CD24^{low}$ and ALDH1 has been studied by various groups using immunohistochemistry (Bane et al., 2013; Currie et al., 2013; Ricardo et al., 2011; Tsang et al., 2012). These studies have typically had small numbers of patient samples, and while some have shown associations with poor tumour characteristics, there have been conflicting results with regards to grade, ER status and age at diagnosis (Currie et al., 2013; Ricardo et al., 2011; Tsang et al., 2012). Additionally, studies have not been able to show an association with patient survival (Bane et al., 2013; Currie et al., 2013), although there are some suggestions that these markers may be associated with survival in a subtype specific manner (Ricardo et al., 2011; Tsang et al., 2012). Interestingly, the results from our NanoString study suggest that CD44 and CD24, might both be upregulated in luminal sphere forming cells, suggesting that perhaps high expression of both markers may be prognostic in luminal tumours. The different genes upregulated in spheres from the different subtypes suggests that there may be certain genes that are characteristic of the sphere-forming cell, which are different in the different subtypes, and this may be the reason for the lack of correlation with patient survival in a cohort of all breast tumours.

## CONCLUSIONS AND FUTURE DIRECTIONS

Through our work with the establishment of patient-derived xenografts (PDX) from primary human breast tumours, we have been able to show that engraftment is correlated with high-grade breast cancers, suggesting that engraftment is a marker of more aggressive breast tumours. While we have not been able to conclusively shown that a primary tumour's ability to engraft is related directly to patient outcome, the association between engraftment and aggressive pathological tumour characteristics suggests this. With longer patient follow-up we may be able to ascertain whether engraftment is significantly related to patient survival.

The quantification of functional tumour initiating cells through limiting dilution assays were performed on a small number of tumours, which were too small to determine a correlation between TIC frequency and patient outcome. However, our study is unique in its approach since it was performed with no pre-sorting of cells based on marker expression in an attempt to elucidate the 'raw' TIC frequency within a tumour, potentially capturing cells that may be missed in the sorting process. The technical difficulty, time, expense and the requirement for fresh tissue are barriers to the adoption of this assay as a clinical tool.

The establishment of spheres from patient tumours was showed little correlation with known adverse tumour pathologic characteristics or patient outcome, although our numbers are small. Additionally, it appears that sphere

forming assays may overestimate the CSC/TIC frequency of a tumour when sphere-forming frequencies (SFF) are compared to functional TICs from LCDT assays. While these assays are technically easier, and more economical to perform than LCDT assays, SFF is not correlated with adverse tumour pathology or patient outcome, and sphere forming assays are unlikely to be clinically useful as a prognostic biomarker.

The expression of CSC genes is able to separate cell lines into luminal and basal subtypes, implying that they may be of prognostic utility. The CSC genes were also able to separate patient tumours into the different breast cancer molecular subtypes, and these subtypes were associated with different patient outcomes. A number of genes were expressed at a level higher in spheres than adherent cells in human breast cancer cell lines and these differentially expressed genes were different depending on the whether the cell line was luminal or basal, suggesting there may be different CSC markers for the different breast cancer subtypes. It remains to be seen whether individual genes are prognostic in patient tumours, or whether a subset of them are prognostic in different molecular subtypes.

When the protein expression of the CSC markers ALDH1, CD44, CD24 and CD49f were examined in a cohort of breast tumours, they were correlated with molecular subtype and a number of aggressive tumour pathological characteristics, such as high tumour grade and large tumour size. Only CD24 was

associated with disease-free patient outcome. The results suggests that the since the expression of these markers is correlated with molecular subtype, they might be prognostic in a subtype specific manner. Further analysis will be required to determine whether they are associated with patient outcome within a molecular subtype.

## TABLES AND FIGURES

**Table 1.** Summary of patient-derived xenografts established from primary human breast tumours.

| Sample Number | Number of cells injected | Route | Time to primary tumour | Number of cells injected | Route | Time to 1st passage | Number of cells injected | Route | Time to 2nd passage | Subtype |
|---|---|---|---|---|---|---|---|---|---|---|
| 30 | 625K | Subcutaneous | 8 months | | Orthotopic | no engraftment | | | | TN |
| 26 | 30K | Subcutaneous | 10 months | 100K | Orthotopic | 3 months | 100K | Subcutaneous | 1.75 months | HER2 |
| 43 | 110K | Orthotopic | 2 months | 100K | Subcutaneous | 1 month | 125K | Subcutaneous | 1 month | TN |
| 76 | 150K | Orthotopic | 14 months | 100K | Subcutaneous | 2.75 months | 450K | Subcutaneous | 2.5 months | LumB |
| 81 | 180K | Orthotopic | 5 months | 100K | Orthotopic | 3.5 months | 1.2M | Subcutaneous | 1.75 months | LumB |
| 84 | 240K | Orthotopic | 6 months | 100K | Subcutaneous | 2.5 months | 800K | Subcutaneous | 2 months | LumB |
| 95 | 150K | Orthotopic | 4 months | 100K | Orthotopic | 1.75 months | 250K | Subcutaneous | 2.25 months | TN |

**Table 2.** Limiting dilution cell transplant assays and sphere forming assays indicating the CSC frequency in patient-derived xenografts

| Study ID # | Xenograft # | Molecular subtype | LDCT assay TIC frequency (95% CI) | Sphere forming frequency |
|---|---|---|---|---|
| 0030 | #12 | TN | 0:231,100 | 1:9,600 |
| 0036 | #26 | HER2 | 1:35,013 (1:194820-1:6293) | 1:755 |
| 0043 | #43 | TN | 1:329 (1:1984-1:54) | 0:≥100,000 |
| 0063 | #63 | Lum B | N/A (not enough tissue) | 1:4,444 |
| 0076 | #76 | Lum B | 1:70,685 (1:471,350-1:10,600) | 0:50,000 |
| 0081 | #81 | Lum B | 1:27,785 (1:154,990-4981) | 1:2,692 |
| 0084 | #84 | TN | 0:121,100 | 1:3,421 |
| 0095 | #95 | Her2 | 1:166,010 (1:1,177,324-1:23,408) | 1:1152 |

**Table 3.** The ability of a tumour to form xenografts as a prognostic factor

| **N** | | No Success 66 | Success 8 | p-value |
|---|---|---|---|---|
| **Age** | *Mean (sd)* | 60.3 (14.4) | 60.3 (13.9) | 0.95 |
| **Molecular Subtype** | *Lum A* | 20 (43.5) | 0 (0.0) | 0.083 |
| | *Lum B* | 14 (30.4) | 3 (37.5) | |
| | *Her2* | 6 (13.0) | 2 (25.0) | |
| | *TN* | 6 (13.0) | 3 (37.5) | |
| | *Missing* | 20 | | |
| **Size of Tumour** | *Median (range)* | 2.8 (0.6, 16.0) | 3.5 (2.5, 8.0) | 0.27 |
| **ER** | *N (%) Positive* | 48 (72.7) | 3 (37.5) | 0.098 |
| **PR** | *N (%) Positive* | 40 (60.6) | 2 (25.0) | 0.070 |
| **HER2** | *N (%) Positive* | 14 (21.2) | 2 (25.0) | 1.00 |
| **Grade** | *1* | 9 (13.6) | 0 (0.0) | **0.009** |
| | *2* | 29 (43.9) | 0 (0.0) | |
| | *3* | 28 (42.4) | 8 (100.0) | |
| **Nodes** | *N (%) Positive* | 65 (98.5) | 7 (87.5) | 0.21 |
| **Lymphvascular Invasion** | *N (%) Present* | 49/64 (76.6) | 5 (62.5) | 0.40 |
| **Extensive In-Situ Component** | *N (%) Present* | 10 (15.2) | 0 (0.0) | 0.59 |
| **Neoadjuvant Treatment** | *N (%) Yes* | 7 (10.6) | 1 (12.5) | 1.00 |
| **Overall Survival** | *N (%) Deaths* | 5 (7.6) | 1 (12.5) | 0.71 |
| | *3-year OS (95% CI)* | 91.2 (77.4, 96.8) | 87.5 (38.7, 98.1) | |
| **Distant Disease-Free Survival** | *N (%) Events* | 6 (9.1) | 2 (25.0) | 0.21 |
| | *3-year DFS (95% CI)* | 90.2 (77.0, 96.0) | 75.0 (31.5, 93.1) | |
| **Event-Free Survival** | *N (%) Events* | 6 (9.1) | 2 (25.0) | 0.22 |
| | *3-year EFS (95% CI)* | 90.6 (78.2, 96.1) | 75.0 (31.5, 93.1) | |

**Table 4.** Primary sphere forming ability as a prognostic factor

| | | No Success | Success | Sphere formation | Sphere forming frequency |
|---|---|---|---|---|---|
| **N** | | 19 | 49 | | |
| **N with Clinical Data** | | 17 | 47 | p-value | p-value |
| **Age** | *Mean (sd)* | 66.0 (13.1) | 59.4 (14.0) | 0.096 | 0.070 |
| **Molecular Subtype** | *Lum A* | 6 (33.3) | 20 (44.4) | 0.71 | 0.26 |
| | *Lum B* | 7 (38.9) | 11 (24.4) | | |
| | *Her2* | 2 (11.1) | 6 (13.3) | | |
| | *TN* | 3 (16.7) | 8 (17.8) | | |
| | *Missing* | 1 | 4 | | |
| **Size of Tumour** | *Median (range)* | 3.5 (1.8, 9.0) | 2.8 (0.6, 16.0) | 0.35 | 0.083 |
| **ER** | *N (%) Positive* | 12 (70.6) | 32 (68.1) | 1.00 | 0.92 |
| **PR** | *N (%) Positive* | 11 (64.7) | 22 (46.8) | 0.26 | 0.66 |
| **HER2** | *N (%) Positive* | 3 (17.7) | 12 (25.5) | 0.74 | 0.86 |
| **Grade** | *1* | 0 (0.0) | 5 (10.6) | 0.16 | 0.32 |
| | *2* | 5 (29.4) | 20 (42.6) | | |
| | *3* | 12 (70.6) | 22 (46.8) | | |
| **Nodes** | *N (%) Positive* | 16 (94.1) | 45 (95.7) | 1.00 | 0.29 |
| **Lymphvascular Invasion** | *N (%) Present* | 12 (70.6) | 33 (73.3) | 1.00 | 0.53 |
| **Extensive In-Situ Component** | *N (%) Present* | 2 (11.8) | 5 (11.1) | 1.00 | 0.75 |
| **Neoadjuvant Treatment** | *N (%) Yes* | 2 (11.8) | 7 (15.2) | 1.00 | 0.99 |
| **Overall Survival** | *N (%) Deaths* | 1 (6.3) | 5 (10.6) | 0.38 | 0.85 |
| | *3-year OS (95% CI)* | 100.0 (-) | 85.8 (68.2, 94.1) | | |
| **Distant Disease-Free Survival** | *N (%) Events* | 3 (18.8) | 5 (10.6) | 0.62 | 0.91 |
| | *3-year DFS (95% CI)* | 87.5 (58.6, 96.7) | 86.7 (70.0, 94.5) | | |
| **Event-Free Survival** | *N (%) Events* | 3 (18.8) | 5 (10.6) | 0.62 | 0.94 |
| | *3-year EFS (95% CI)* | 87.5 (58.6, 96.7) | 87.4 (71.8, 94.7) | | |

**Table 5.** Secondary sphere forming ability as a prognostic factor

| | | No Success | Success | p-value |
|---|---|---|---|---|
| **N** | | 40 | 9 | |
| **Age** | *Mean (sd)* | 50.2 (14.7) | 60.1 (11.8) | 0.83 |
| **Molecular Subtype** | *Lum A* | 14 (38.9) | 6 (66.7) | 0.35 |
| | *Lum B* | 9 (25.0) | 2 (22.2) | |
| | *Her2* | 5 (13.9) | 1 (11.1) | |
| | *TN* | 8 (22.2) | 0 (0.0) | |
| | *Missing* | 4 | | |
| **Size of Tumour** | *Median (range)* | 2.6 (0.7, 16.0) | 4.5 (0.6, 13.5) | 0.088 |
| **ER** | *N (%) Positive* | 24/38 (63.2) | 8 (88.9) | 0.24 |
| **PR** | *N (%) Positive* | 16/38 (42.1) | 6 (66.7) | 0.27 |
| **HER2** | *N (%) Positive* | 11/38 (29.0) | 1 (11.1) | 0.41 |
| **Grade** | *1* | 3 (7.9) | 2 (22.2) | 0.40 |
| | *2* | 16 (42.1) | 4 (44.4) | |
| | *3* | 19 (50.0) | 3 (33.3) | |
| **Nodes** | *N (%) Positive* | 36 (94.7) | 9 (100.0) | 1.00 |
| **Lymphvascular Invasion** | *N (%) Present* | 26/37 (70.3) | 7/8 (87.5) | 0.42 |
| **Extensive In-Situ Component** | *N (%) Present* | 4/36 (11.1) | 1 (11.1) | 1.00 |
| **Neoadjuvant Treatment** | *N (%) Yes* | 4 (10.8) | 3 (33.3) | 0.12 |
| **Overall Survival** | *N (%) Deaths* | 5 (13.2) | 0 (0.0) | 0.21 |
| | *3-year OS (95% CI)* | 81.3 (58.7, 92.3) | 100.0 (-) | |
| **Distant Disease-Free Survival** | *N (%) Events* | 5 (13.2) | 0 (0.0) | 0.21 |
| | *3-year DFS (95% CI)* | 82.3 (60.0, 92.8) | 100.0 (-) | |
| **Event-Free Survival** | *N (%) Events* | 5 (13.2) | 0 (0.0) | 0.22 |
| | *3-year EFS (95% CI)* | 83.7 (64.0, 93.1) | 100.0 (-) | |

**Table 6.** IHC expression of CSC markers compared to tumour pathological characteristics.

| | | ALDH-1 | | CD44 | | CD24 | | CD49f | |
|---|---|---|---|---|---|---|---|---|---|
| | | **N (%) ≥5** | **p-value** | **N (%) ≥5** | **p-value** | **N (%) ≥5** | **p-value** | **N (%) ≥5** | **p-value** |
| **All Patients** | | 67 (8.9) | | 562 (70.6) | | 326 (47.0) | | 203 (24.9) | |
| **Molecular** | *Luminal A* | 15 (4.3) | **<0.001** | 239 (65.1) | **<0.001** | 134 (43.5) | **<0.001** | 61 (15.8) | **<0.001** |
| **Subtype** | *Luminal B* | 20 (8.4) | | 180 (73.5) | | 127 (56.4) | | 53 (21.2) | |
| | *Her2E* | 6 (17.7) | | 28 (75.7) | | 24 (75.0) | | 15 (42.9) | |
| | *Basal* | 22 (21.0) | | 92 (84.4) | | 30 (29.4) | | 69 (60.0) | |
| | *Other* | 4 (12.9) | | 23 (60.5) | | 11 (40.7) | | 5 (15.6) | |
| **Treatment** | *50 Gy* | 27 (7.4) | 0.20 | 289 (73.5) | 0.074 | 158 (45.8) | 0.54 | 99 (25.0) | 0.94 |
| **Arm** | *42.5 Gy* | 40 (10.2) | | 273 (67.7) | | 168 (48.1) | | 104 (24.7) | |
| **Age** | *<50* | 30 (14.4) | **0.001** | 159 (72.9) | 0.38 | 77 (39.5) | **0.014** | 75 (33.2) | **<0.001** |
| | *≥50* | 37 (6.8) | | 403 (69.7) | | 249 (49.9) | | 128 (21.7) | |
| **Size** | *≤2 cm* | 37 (6.4) | **<0.001** | 433 (69.7) | 0.35 | 231 (44.0) | **0.006** | 145 (22.8) | **0.015** |
| | *>2 cm* | 30 (17.2) | | 129 (73.7) | | 95 (56.2) | | 58 (32.0) | |
| **Nottingham** | *I* | 4 (3.3) | **<0.001** | 94 (68.1) | 0.062 | 41 (40.6) | 0.20 | 21 (15.8) | **<0.001** |
| **Grade** | *II* | 27 (6.9) | | 277 (69.1) | | 184 (50.7) | | 70 (17.0) | |
| | *III* | 23 (15.3) | | 118 (78.7) | | 68 (48.2) | | 75 (48.4) | |

**Table 7.** IHC expression of CSC markers as a prognostic factor for patient outcome

| | | Local Recurrence | | Overall Survival | | Disease-Free Survival | |
|---|---|---|---|---|---|---|---|
| | | **Hazards Ratio (95% CI)** | **p-value** | **Hazards Ratio (95% CI)** | **p-value** | **Hazards Ratio (95% CI)** | **p-value** |
| **ALDH-1** | *≥5* | 1.70 (0.77-3.77) | 0.19 | 0.74 (0.40-1.37) | 0.34 | 0.92 (0.57-1.49) | 0.74 |
| **CD44** | *≥5* | 1.63 (0.84-3.16) | 0.15 | 0.90 (0.65-1.24) | 0.53 | 1.02 (0.77-1.35) | 0.90 |
| **CD24** | *≥5* | 1.20 (0.70-2.06) | 0.50 | 1.27 (0.92-1.74) | 0.15 | 1.45 (1.11-1.91) | **0.008** |
| **CD49f** | *≥5* | 1.11 (0.60-2.03) | 0.75 | 0.91 (0.64-1.30) | 0.60 | 0.91 (0.67-1.24) | 0.55 |

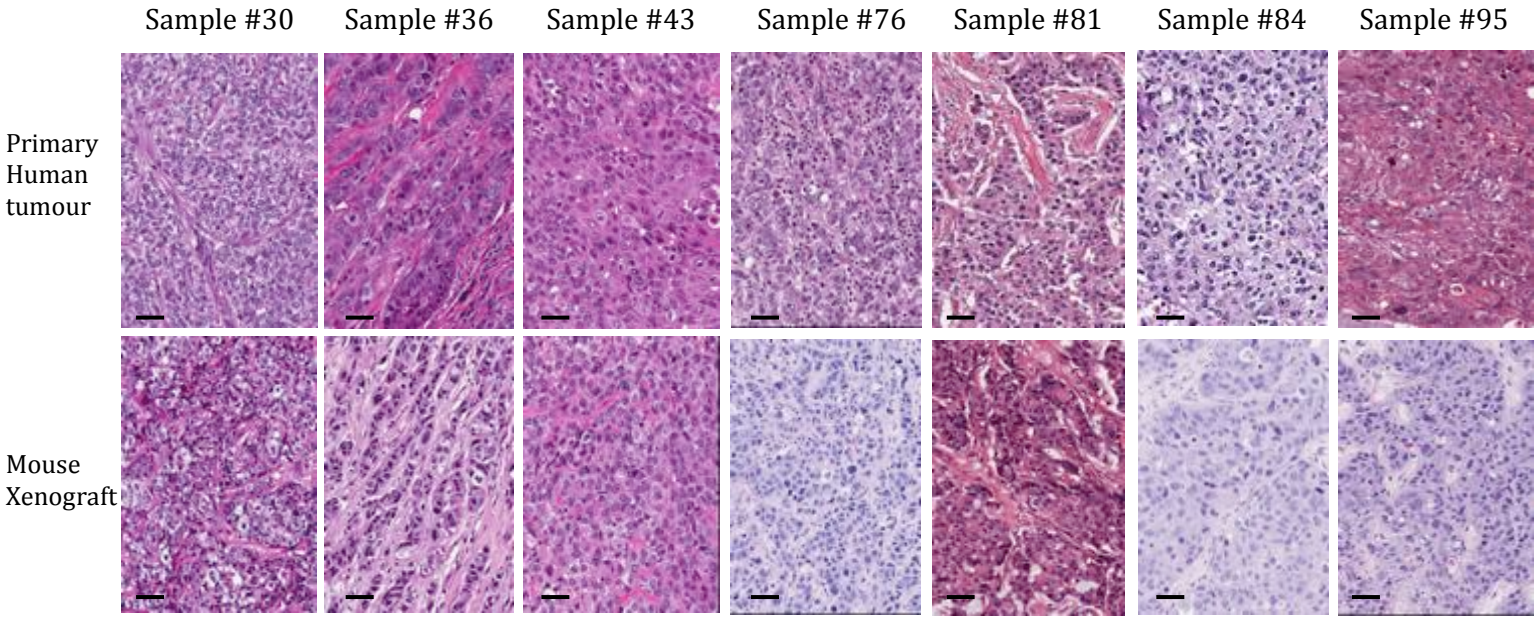
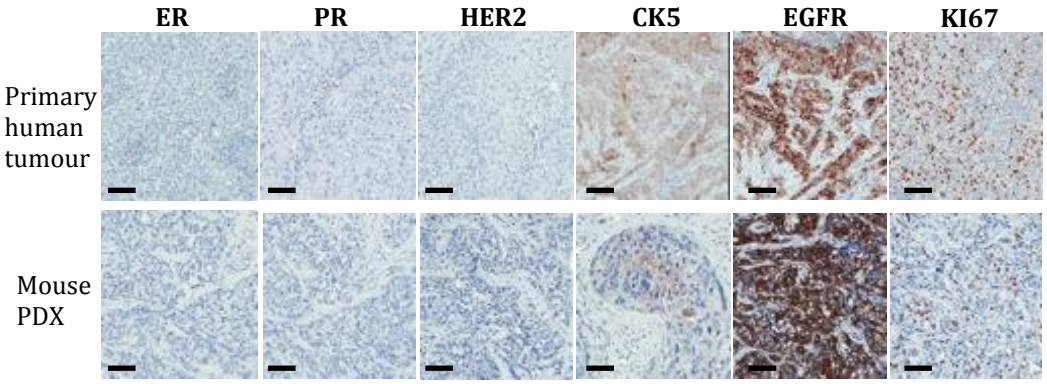
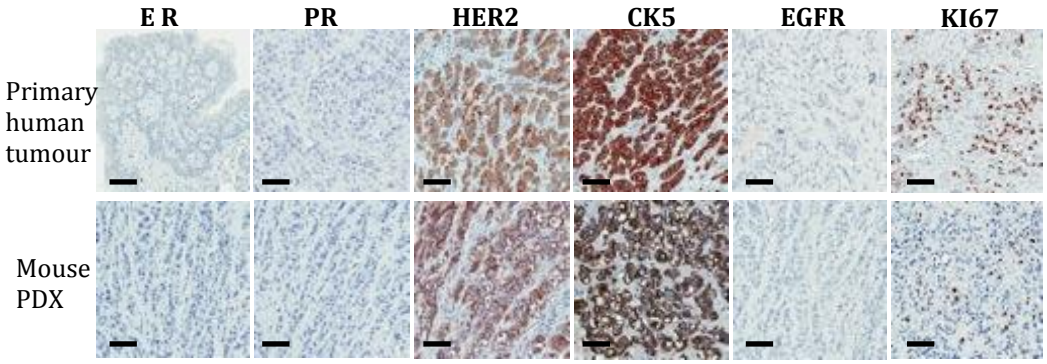



**Figure 1.** H&E staining of primary human tumours and corresponding patient-derived xenografts (PDX). Images are representative sections of each tumour stained with hematoxylin and eosin (H&E) at 20x. Scale bars represent 100μm.

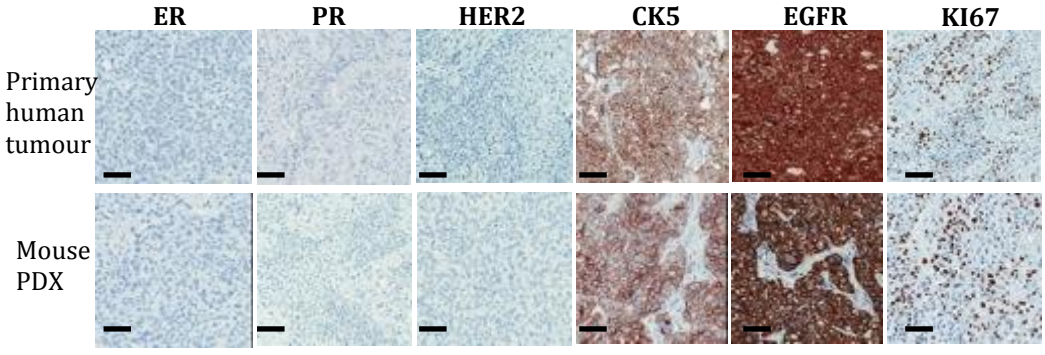
**A. Sample #30**



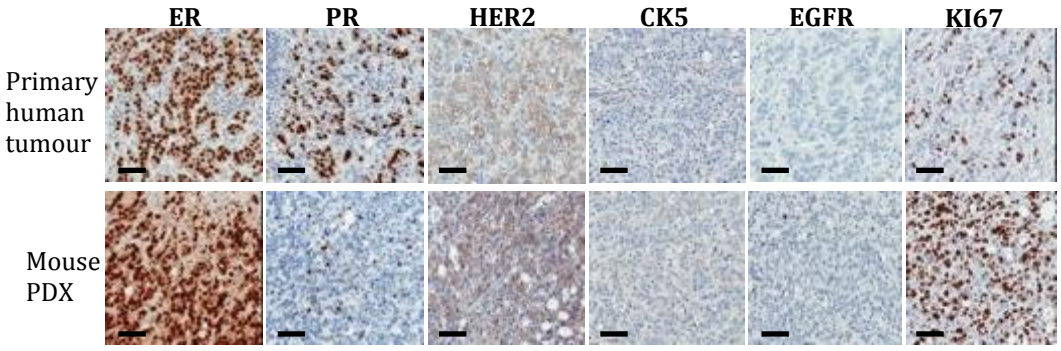

**B. Sample #36**



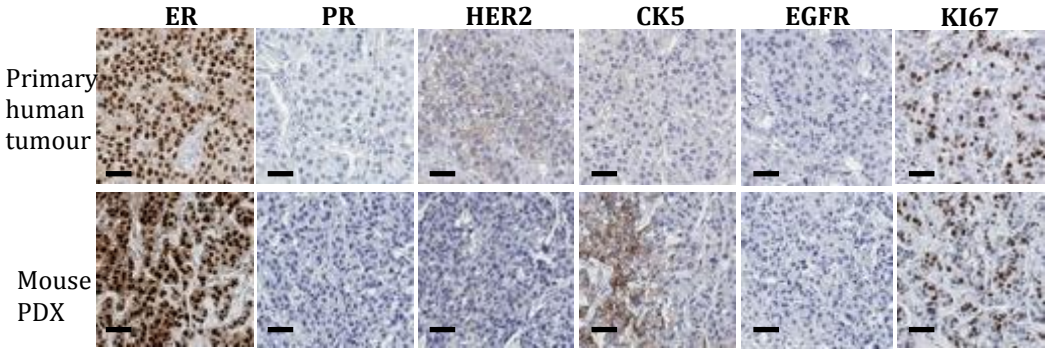

**C. Sample #43**



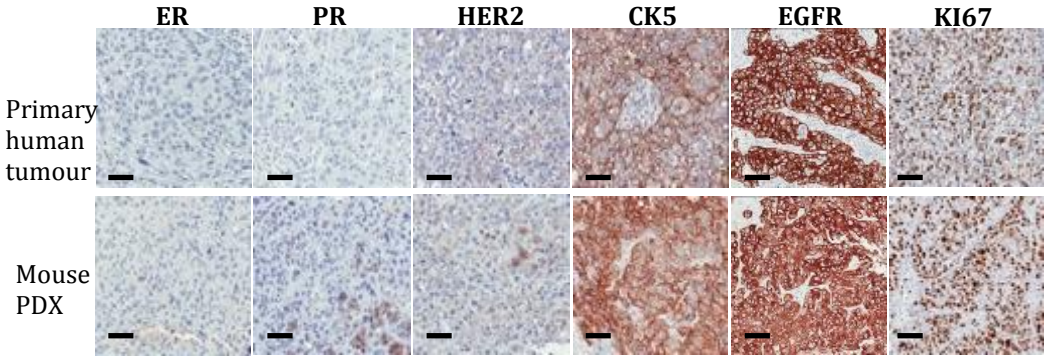
**D. Sample #76**




**E. Sample #81**




**F. Sample #84**



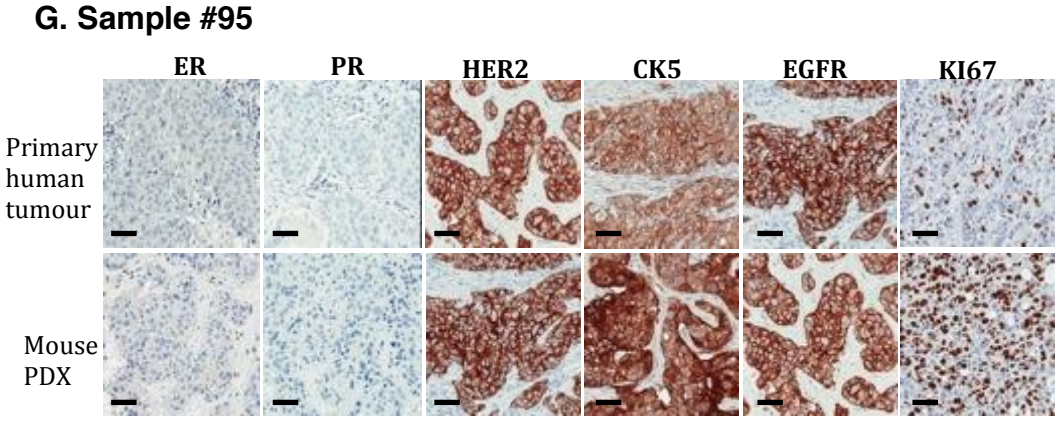



**Figure 2.** IHC staining of primary human tumours and corresponding patient-derived xenografts. Immunohistochemistry for a panel of clinical markers. Images are of representative sections of tumours taken at 20x. Scale bars represent 100µm.

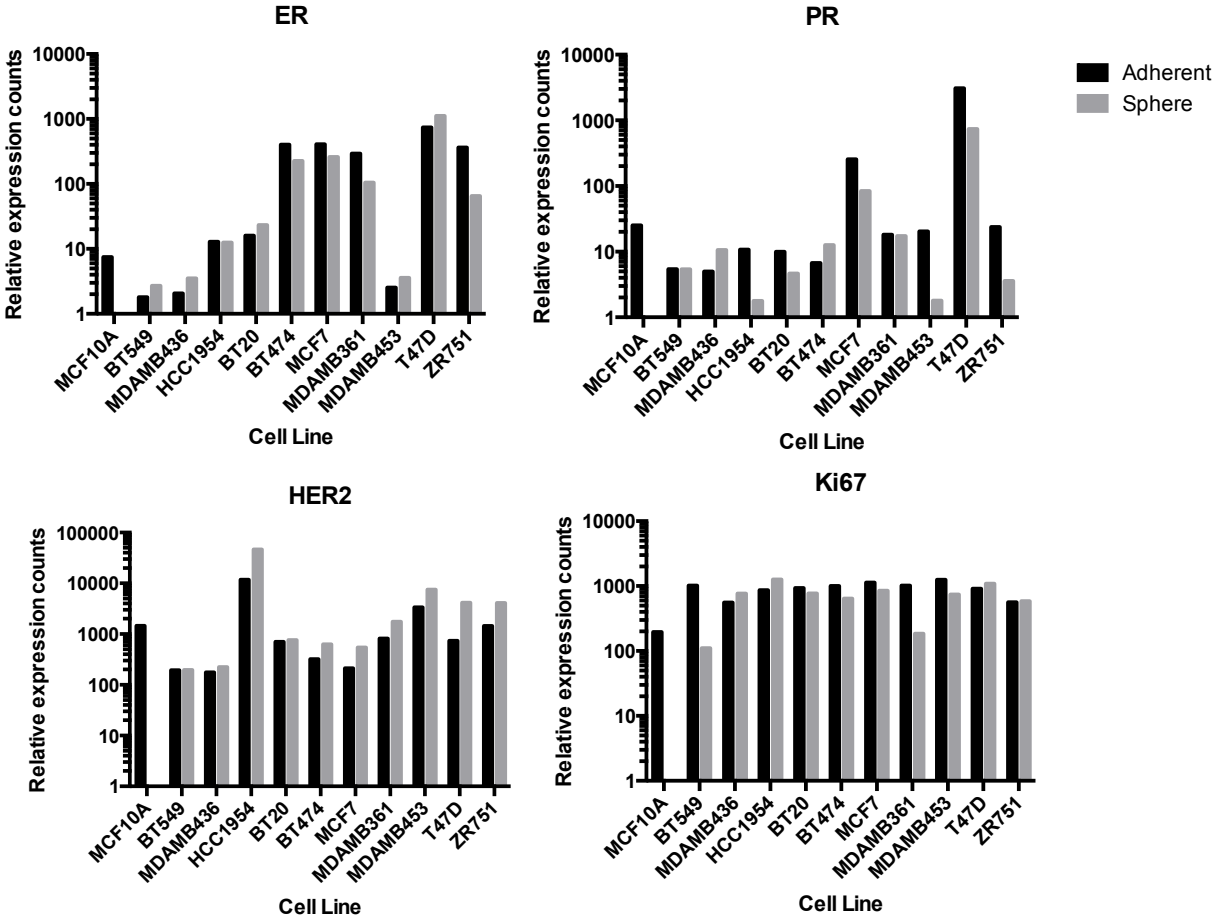



**Figure 3.** Relative expression counts of clinical markers ER, PR HER2 and Ki67 using NanoString probes in human breast cancer cell lines grown adherently and as spheres.

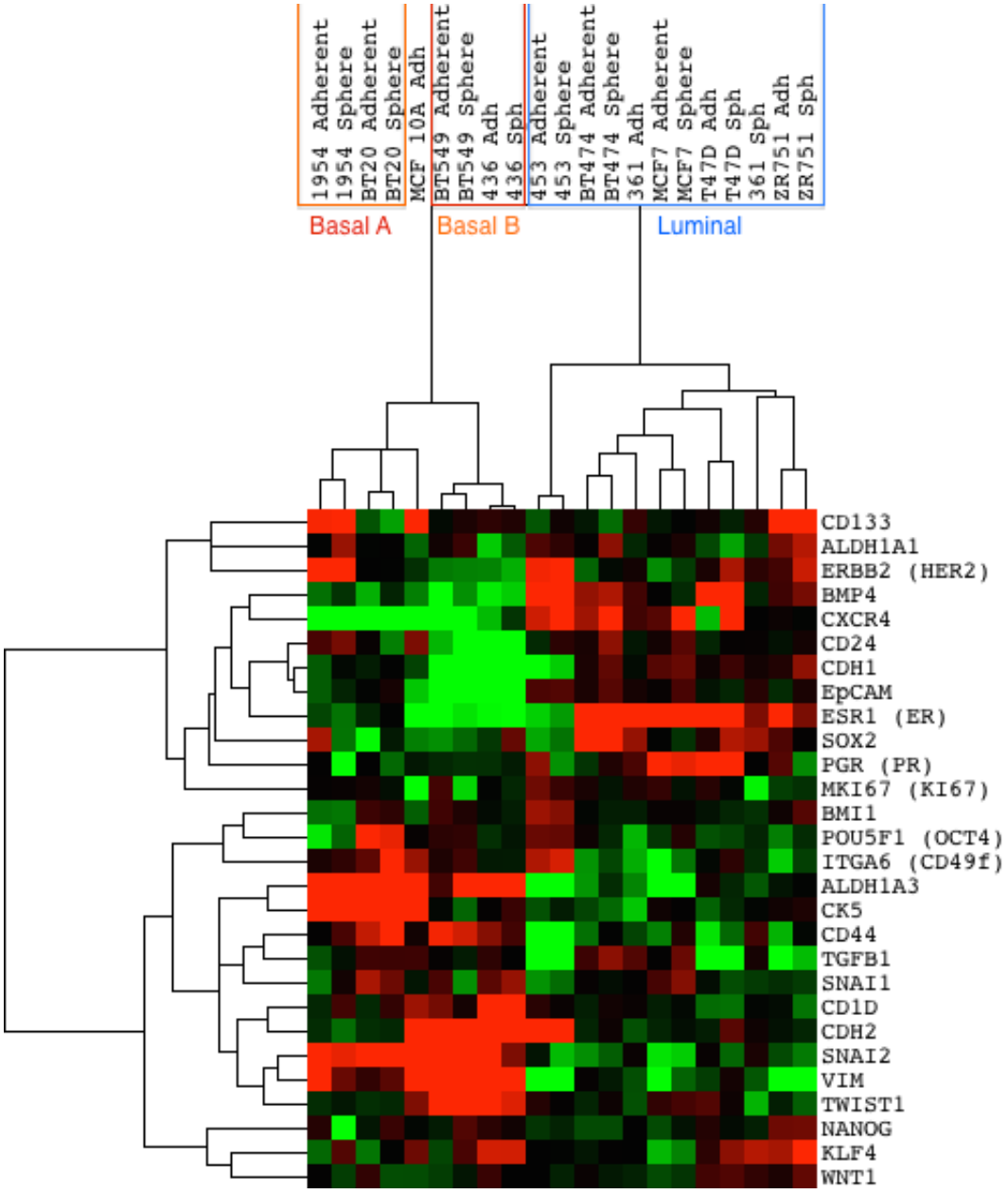



**Figure 4.** Heatmap and cluster diagram of cell lines using expression of CSC genes. Red indicates high expression and green indicates low expression Cell lines cluster by subtype into luminal, basal A and basal B clusters.

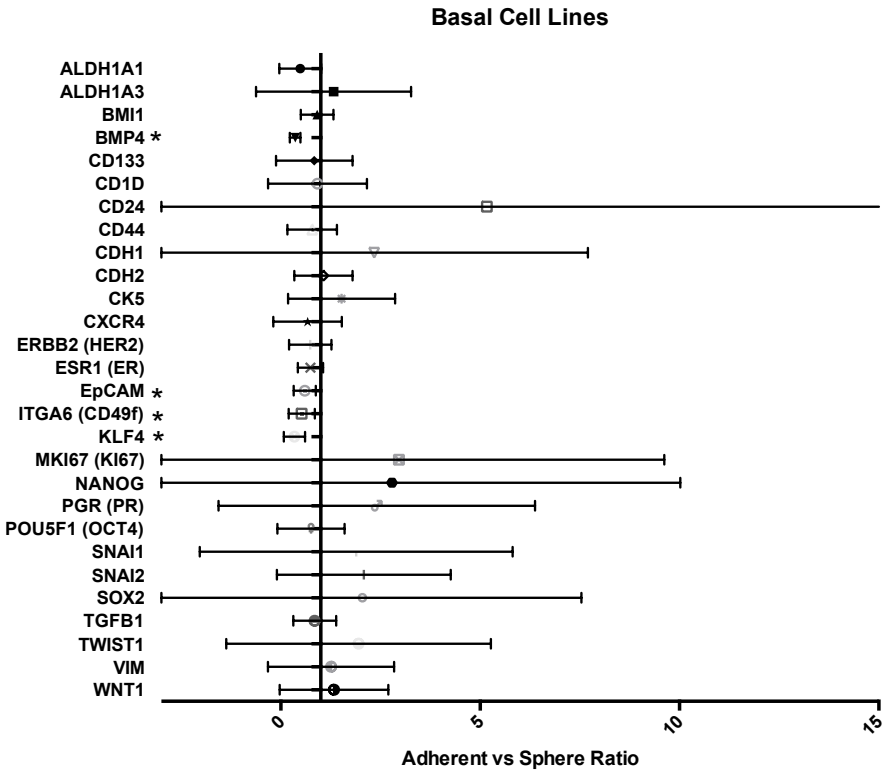



**Figure 5.** 95% confidence intervals for adherent vs sphere ratios for each CSC gene in basal breast cancer cell lines (basal A and basal B combined). *CI95 that are less than 1 indicate significantly higher expression in spheres than adherent cells.

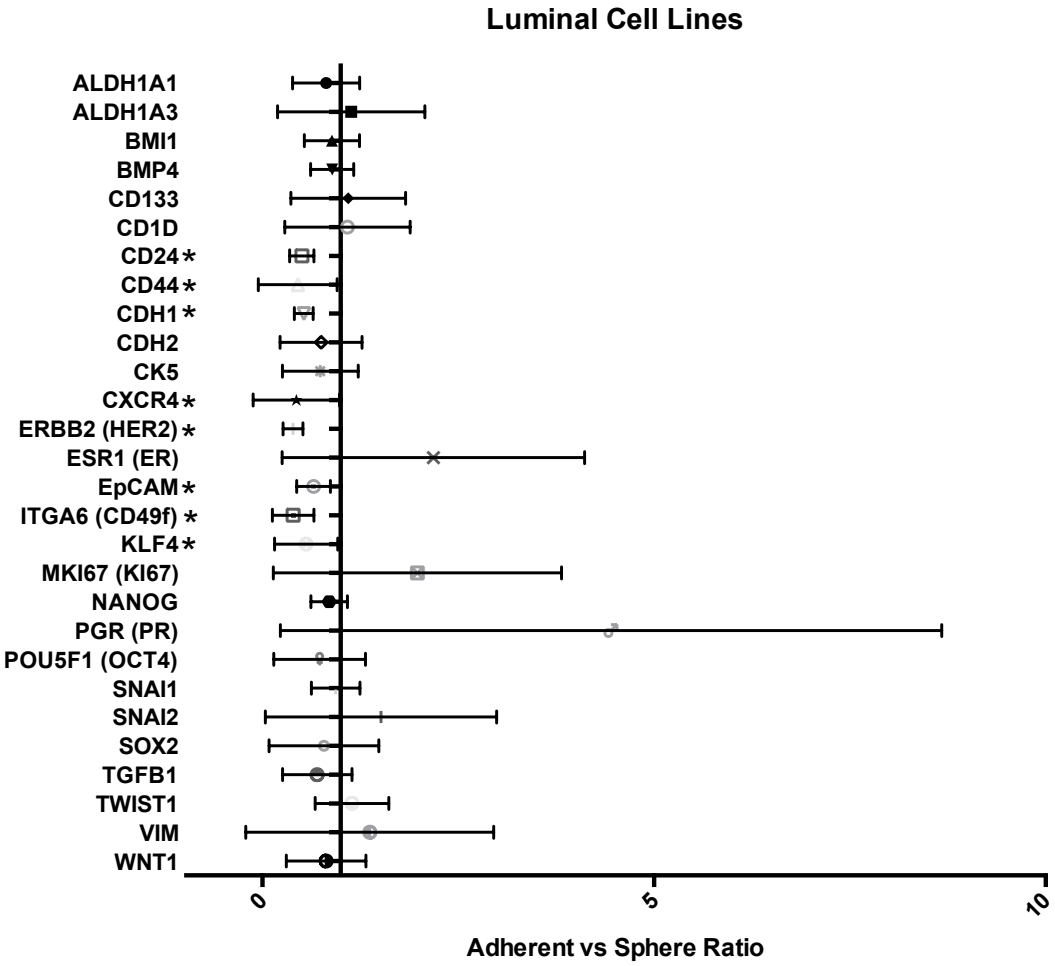



**Figure 6.** 95% confidence intervals for adherent vs sphere ratios for each CSC gene in luminal breast cancer cell lines. *CI95 that are less than 1 indicate significantly higher expression in spheres than adherent cells.

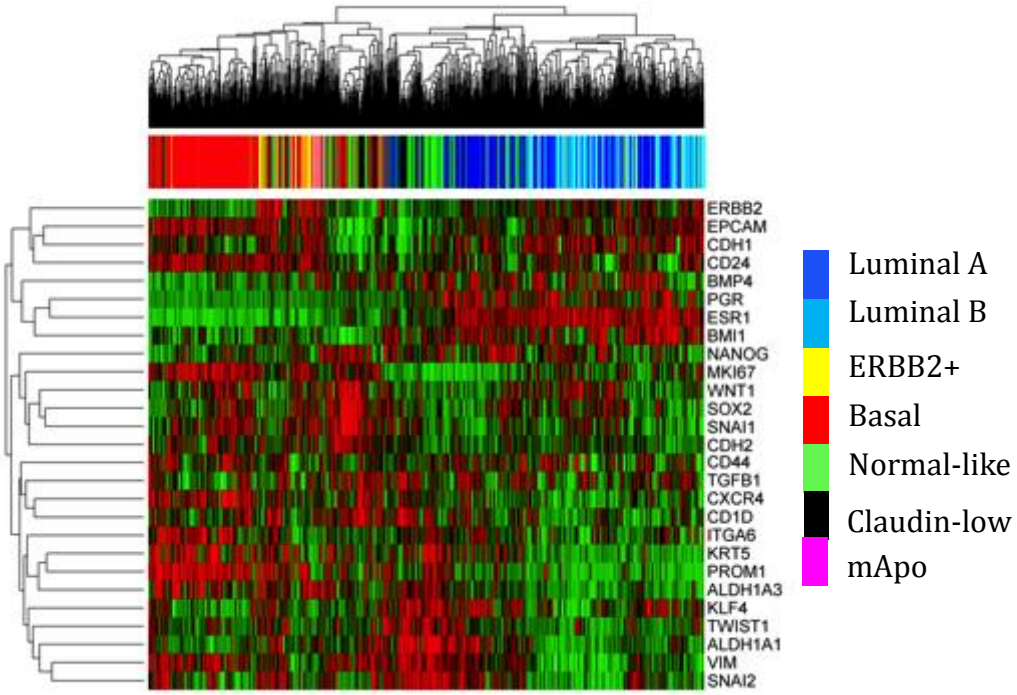



**Figure 7.** Heatmap of a cohort of 1, 593 primary breast tumours clustered using the expression CSC genes in silico from microarray data on the Affymetrix GeneChip HG-U133A. The gene signature is able to broadly classify tumours into luminal and basal subtypes.

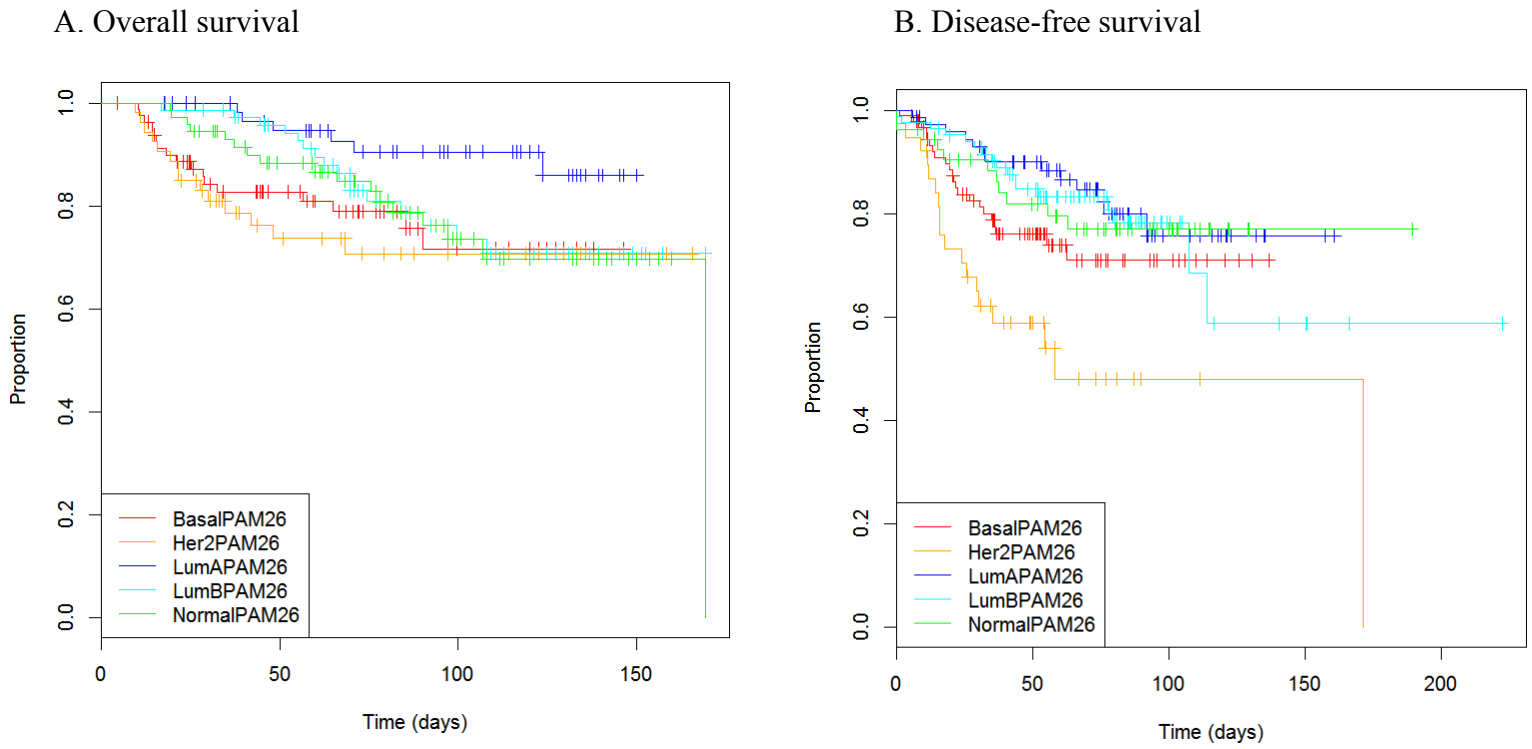



**Figure 8.** (A) Overall survival and (B) Disease-free survival of 1,593 breast cancer patients separated by subtypes generated by the CSC genes.

**Supplementary Table 1.** Cell lines and adherent culture conditions

| Cell Line | Subtype | Culture Media | Culture Conditions |
|---|---|---|---|
| MCF10A | | DMEM/F12 | 37°C, 5% CO2 |
| MCF7 | Luminal | DMEM, 10%FBS | 37°C, 5% CO2 |
| HCC1954 | Basal A (HER2+) | RPMI, 10% FBS | 37°C, 5% CO2 |
| BT474 | Luminal | RPMI, 10% FBS | 37°C, 5% CO2 |
| BT549 | Basal B | RPMI, 10% FBS | 37°C, 5% CO2 |
| MDAMB361 | Luminal | DMEM, 10%FBS | 37°C, 5% CO2 |
| MDAMB453 | Luminal | DMEM, 10%FBS | 37°C, 5% CO2 |
| BT20 | Basal A | DMEM, 10%FBS | 37°C, 5% CO2 |
| ZR751 | Luminal | RPMI, 10% FBS | 37°C, 5% CO2 |
| T47D | Luminal | RPMI, 10% FBS | 37°C, 5% CO2 |

**Supplementary Table 2.** Candidate CSC genes for NanoString probe construction

| Name | Gene | Marker |
|---|---|---|
| Aldehyde dehydrogenase 1A1 | ALDH1A1 | |
| Aldehyde dehydrogenase 1A3 | ALDH1A3 | |
| Polycomb ring finger oncogene | BMI1 | |
| Bone morphogenic protein 4 | BMP4 | |
| CD1D | CD1D | |
| CD24 | CD24 | |
| CD44 | CD44 | |
| E-cadherin | CDH1 | |
| N-cadherin | CDH2 | |
| C-X-C chemokine receptor type 4 | CXCR4 | |
| Epithelial cell adhesion molecule | EpCAM | |
| Human epidermal growth factor receptor 2 | ERBB2 (HER2) | Clinical marker |
| Estrogen receptor | ER | Clinical marker |
| Integrin alpha 6 | ITGA6 (CD49f) | |
| Kruppel-like factor 4 | KLF4 | |
| Cytokeratin 5 | CK5 | |
| Marker of proliferarion Ki67 | KI67 | Clinical marker |
| Nanog homeobox | NANOG | |
| Progesterone receptor | PR | Clinical marker |
| Octamer binding transcription factor 4 | OCT4 | |
| Prominin 1 | CD133 | |
| Snail family zinc finger 1 | SNAI1 | |
| Snail family zinc finger 2 (Slug) | SNAI2 | |
| Sex determining region Y-box 2 | SOX2 | |
| Transforming growth factor B1 | TGFB1 | |
| Twist-realted protein 1 | TWIST1 | |
| Vimentin | VIM | |
| Wingless-type MMTV integration site family, member 1 | WNT1 | |
| Beta actin | ACTB | Housekeeping |
| Glyceraldehyde-3-phosphate dehydrogenase | GAPDH | Housekeeping |
| Beta glucuronidase | GUSB | Housekeeping |
| Hypoxanthine phosphoribosyltransferase 1 | HPRT1 | Housekeeping |
| Phosphoglycerate kinase 1 | PGK1 | Housekeeping |

**Supplementary Table 3.** Limiting dilution cell transplant analysis for successful xenografts (0/2 indicates 0 tumours formed when 2 mice were injected).

| Sample # | Number of cells injected | | | | TIC frequency |
|---|---|---|---|---|---|
| | 100,000 | 10,000 | 1,000 | 100 | |
| 12 | 0/2 | 0/3 | 0/1 | 0/1 | 0:231,100 |
| 26 | 2/2 | 0/1 | 0/2 | 0/2 | 1:35,013 |
| 43 | 2/2 | 2/2 | 2/2 | 0/1 | 1:329 |
| 76 | 1/1 | 0/3 | 0/2 | 0/1 | 1:70,685 |
| 81 | 1/1 | 0/3 | 1/1 | 0/1 | 1:27,785 |
| 84 | 0/1 | 0/2 | 0/1 | 0/1 | 0:121,100 |
| 95 | 1/2 | 0/2 | 0/1 | | 1:166,010 |

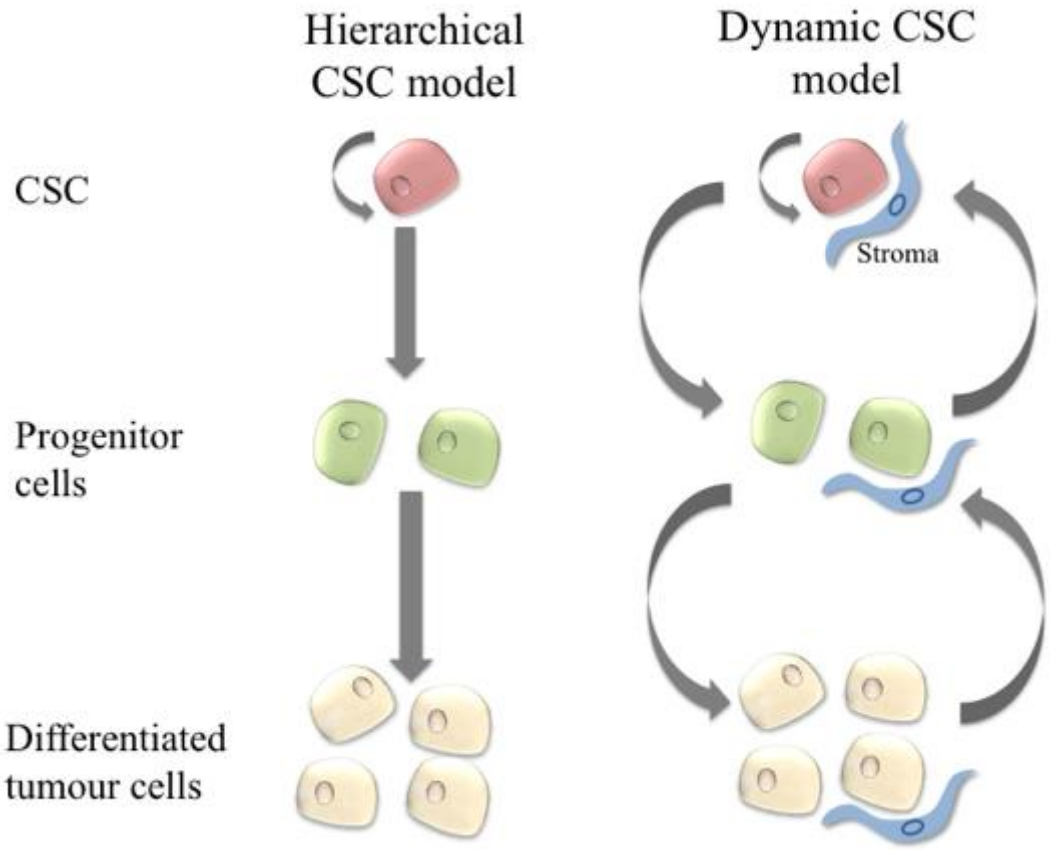
**Supplementary Table 4.** Primary and secondary sphere forming assays for primary breast tumours.

| Sample # | Primary SFF | Secondary SFF |
|---|---|---|
| 2 | 1;2,500 | |
| 3 | 0:≥100,000 | |
| 10 | 0:≥100,000 | |
| 11 | 1:1,545 | |
| 12 | 1:3,333 | |
| 14 | 1:8,333 | |
| 15 | 0:≥100,000 | |
| 21 | 0:≥100,000 | |
| 22 | 0:≥100,000 | |
| 23 | 0:≥100,000 | |
| 24 | 0:≥100,000 | |
| 26 | 0:≥100,000 | |
| 30 | 1:9,600 | |
| 34 | 1:14,000 | |
| 36 | 0.565972222 | |
| 43 | 0:≥100,000 | |
| 47 | 1:2,857 | |
| 48 | 1:13,333 | |
| 50 | 1:870 | 1:533 |
| 51 | 1:22,222 | 1:12,000 |
| 52 | 1:30,769 | 0:7,500 |
| 54 | 1:2,273 | |
| 55 | 1:2,073 | 1:3,900 |
| 56 | 0:≥100,000 | |
| 58 | 0:20,000 | |
| 61 | 0:≥100,000 | |
| 62 | 1:12,087 | |
| 63 | 1:4,444 | 1:6,000 |
| 64 | | |
| 66 | 1:1,736 | |
| 67 | 1:536 | |
| 69 | 1:3,500 | 1:1,200 |
| 71 | 1:1,961 | |

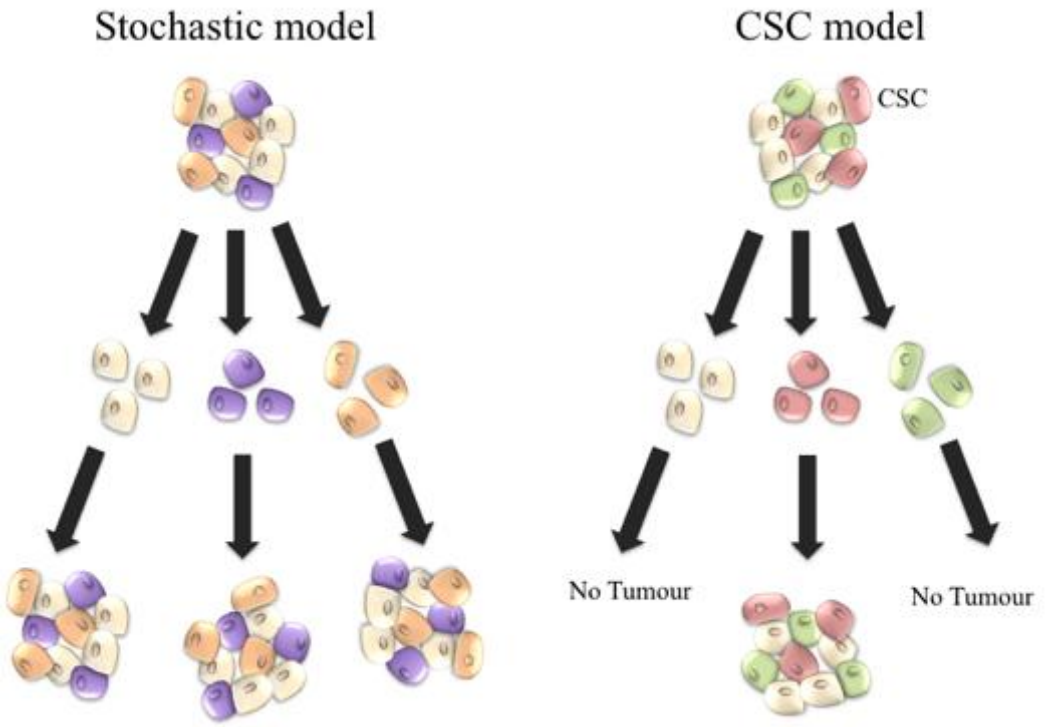
| | | |
|---|---|---|
| 72 | 0:30,000 | |
| 73 | 1:490 | 1:346 |
| 74 | 0:2,500 | |
| 75 | 1:804 | |
| 76 | 0:50,000 | |
| 78 | 1:2,763 | |
| 80 | 1:3,077 | |
| 81 | 1:2,692 | |
| 83 | 1:6,667 | 1:1,857 |
| 84 | 1:3,421 | |
| 85 | 0:20,000 | |
| 86 | 1:30,000 | |
| 89 | 1:2,439 | |
| 91 | 0:20,000 | |
| 92 | 0:10,000 | |
| 94 | 1:2,927 | |
| 95 | 1:1,152 | |
| 98 | 1:10,000 | |
| 99 | 1:11,000 | |
| 100 | 1:9,375 | |
| 102 | 1:1,667 | |
| 106 | 1:2,532 | |
| 107 | 1:4,545 | |
| 113.1 | 1:692 | |
| 113.2 | 1:6,250 | |
| 119R | 1:1,613 | |
| 119L | 1:2,105 | |
| 121 | 1:8,333 | |
| 123 | 1:1,884 | |
| 124 | 1:608 | |
| 125 | 1:1,222 | |
| 130 | 1:3,000 | |
| 134 | 1:1,307 | 1:1,875 |
| 136 | 1:2,619 | |
| 138 | 1:1,500 | 1:15,000 |

**Supplementary Table 5.** Overall clinical and survival characteristics for xenograft study patients

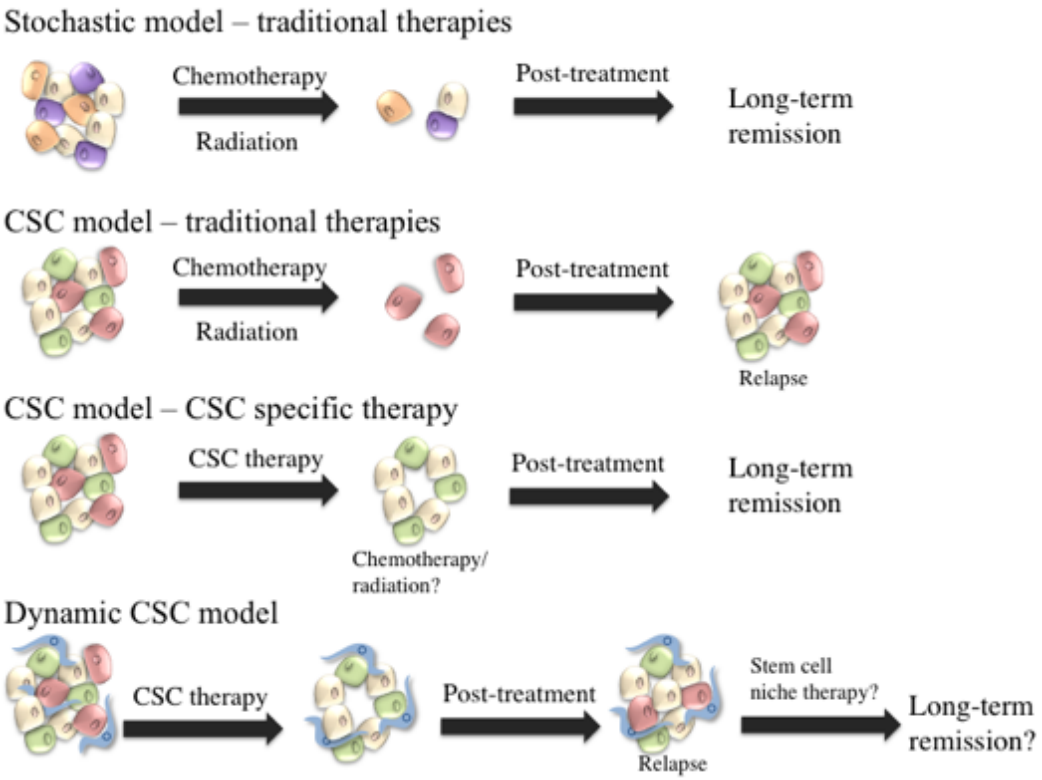
| | | | |
|---|---|---|---|
| **Age** | *Mean (sd)* | *N=74* | 60.3 (14.3) |
| **Molecular Subtype** | *Lum A* | *54* | 20 (37.0) |
| | *Lum B* | | 17 (31.5) |
| | *Her2* | | 8 (14.8) |
| | *TN* | | 9 (16.7) |
| **Breast** | *N (%) Left* | *74* | 29 (39.2) |
| **Surgery** | *N (%) with BCS* | *74* | 32 (43.2) |
| **Tumour Type** | *Carcinoma Unspecified* | *74* | 1 (1.4) |
| | *DCIS* | | 1 (1.4) |
| | *Invasive Mucinous Carcinoma* | | 2 (2.7) |
| | *Invasive w/ductal & lobular* | | 2 (2.7) |
| | *Invasive Ductal* | | 57 (77.0) |
| | *Invasive Lobular* | | 10 (13.5) |
| | *Mucinous Carcinoma* | | 1 (1.4) |
| **Size of Tumour** | *Median (range)* | *72* | 2.9 (0.6, 16.0) |
| **ER** | *N (%) Positive* | *74* | 51 (68.9) |
| **PR** | *N (%) Positive* | *74* | 42 (56.8) |
| **HER2** | *N (%) Positive* | *74* | 16 (21.6) |
| **Grade** | *1* | *74* | 9 (12.2) |
| | *2* | | 29 (39.2) |
| | *3* | | 36 (48.7) |
| **Stage** | *0* | *71* | 1 (1.4) |
| | *I* | | 8 (11.3) |
| | *II* | | 43 (60.6) |
| | *III* | | 19 (26.8) |
| **Nodes** | *N (%) Positive* | *74* | 72 (97.3) |
| **Number of Nodes Removed** | *Median (range)* | *72* | 12 (1, 29) |
| **Number of Nodes Positive** | *Median (range)* | *72* | 0 (0, 22) |
| **% of Nodes Positive** | *Median (range)* | *72* | 0 (0, 100) |
| **Margins** | *N (%) Positive* | *74* | 3 (4.1) |
| **Lymphvascular Invasion** | *N (%) Present* | *72* | 54 (75.0) |
| **Extensive In-Situ Compon.** | *N (%) Present* | *72* | 10 (13.9) |
| **Neoadjuvant Treatment** | *N (%) Yes* | *74* | 8 (10.8) |
| **Adjuvant Treatment** | *N (%) Yes* | *74* | 43 (48.1) |
| **Hormone Treatment** | *N (%) Yes* | *74* | 47 (63.5) |
| **Herceptin** | *N (%) Yes* | *74* | 17 (23.0) |
| **Radiation Treatment** | *N (%) Yes* | *74* | 47 (63.5) |
| **Boost** | *N (%) Yes* | *74* | 10 (13.5) |
| **Regional Nodes Radiated** | *N (%) Yes* | *74* | 28 (37.8) |
| | **Outcomes** | | |
| **Xenograft** | *N (%) Successful* | *74* | 8 (10.8) |
| **Overall Survival** | *N (%) Deaths* | *74* | 6 (8.1) |
| | *3-year OS (95% CI)* | | 90.8 (78.8, 96.2) |
| **Distant Disease-Free** | *N (%) Events* | *74* | 8 (10.8) |
| **Survival** | *3-year DFS (95% CI)* | | 88.4 (76.6, 94.5) |
| **Event-Free Survival** | *N (%) Events* | *74* | 8 (10.8) |
| | *3-year EFS (95% CI)* | | 88.7 (77.5, 94.6) |



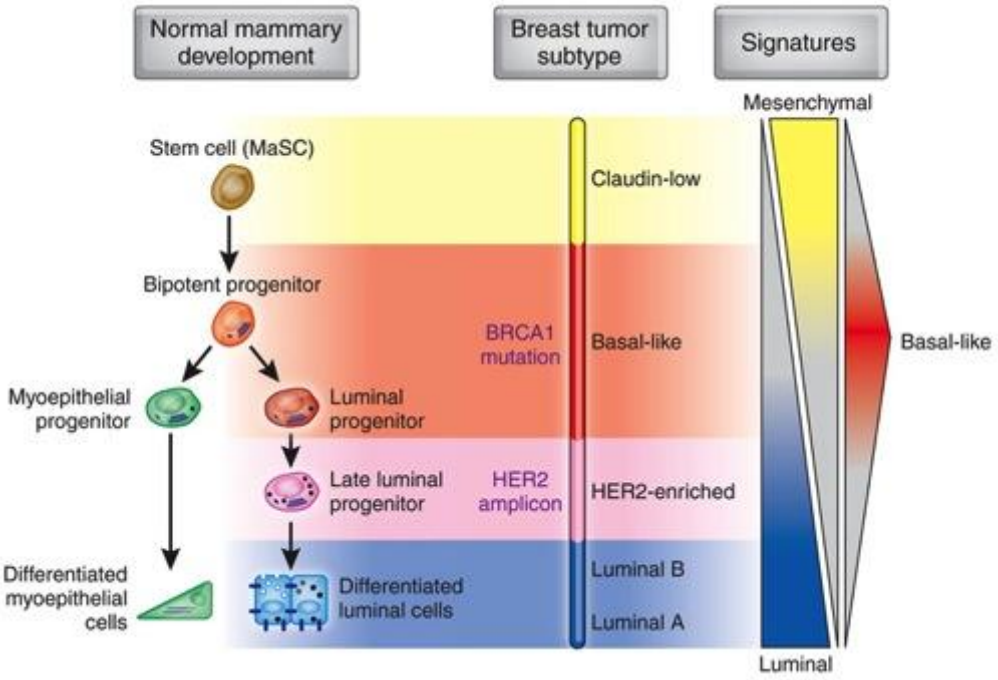

**Supplementary Figure 1.** The hierarchical model and dynamic model of CSC tumour development. In the hierarchical model, CSCs have the ability to self-renew and give rise to more differentiated progeny in a unidirectional manner. In the dynamic model, more differentiated progeny are able to de-differentiate in response to stimuli from the stem cell nice. Adapted from Vermeulen et al., 2012.



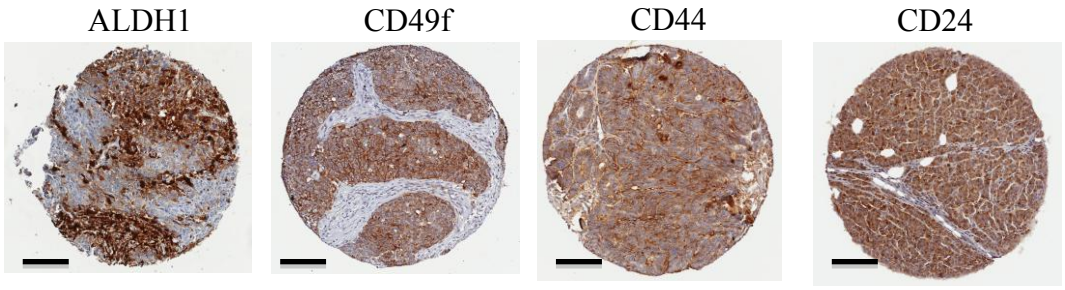

**Supplementary Figure 2.** The stochastic model of tumourigenesis and the CSC model of tumourigenesis. In the stochastic model, all tumours have an equal probability of seeding a new tumour. In the CSC model, the CSC is the only cell able to give rise to tumours whereas the bulk of the tumour is non-tumourigenic.




**Supplementary Figure 3.** Cancer therapeutics and the different models of tumour development. Traditional therapies may be ineffective against CSC and lead to relapse post-treatment. Under the CSC model a CSC specific therapy would target CSCs and in combination with traditional therapies may achieve durable remission. Under the dynamic CSC model, more differentiated cells may survive and de-differentiate into CSCs and seed relapses, suggesting a need for an additional stem cell niche therapy to achieve long-term remission. Adapted from Vermeulen et al., 2012.




**Supplementary Figure 4.** Normal mammary epithelial stem and progenitor cells and their relationship with breast cancer molecular subtypes. The different molecular subtypes of breast cancer may arise from transformation of specific stem and/or progenitor cells within the normal mammary epithelial cell hierarchy. Figure from Prat and Perou, 2009.




**Supplementary Figure 5.** Representative immunohistochemical staining of ALDH1, CD49f, CD44 and CD24 on sections from tissue microarray cores. Scale bars represent 100μM.

## REFERENCES

Adams, E. J., & López-Sagaseta, J. (2011). The immutable recognition of CD1d. *Immunity*, *34*(3), 281–3.

Al-Hajj, M., Wicha, M. S., Benito-Hernandez, A., Morrison, S. J., & Clarke, M. F. (2003). Prospective identification of tumorigenic breast cancer cells. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(7), 3983–8.

Alarmo, E.-L., Kuukasjärvi, T., Karhu, R., & Kallioniemi, A. (2007). A comprehensive expression survey of bone morphogenetic proteins in breast cancer highlights the importance of BMP4 and BMP7. *Breast Cancer Research and Treatment*, *103*(2), 239–46.

Albain, K. S., Barlow, W. E., Shak, S., Hortobagyi, G. N., Livingston, R. B., Yeh, I.-T., … Hayes, D. F. (2010). Prognostic and predictive value of the 21-gene recurrence score assay in postmenopausal women with node-positive, oestrogen-receptor-positive breast cancer on chemotherapy: a retrospective analysis of a randomised trial. *The Lancet Oncology*, *11*(1), 55–65.

Allred, D. C., Harvey, J. M., Berardo, M., & Clark, G. M. (1998). Prognostic and predictive factors in breast cancer by immunohistochemical analysis. *Modern Pathology*, *11*(2), 155–68.

Aruffo, a, Stamenkovic, I., Melnick, M., Underhill, C. B., & Seed, B. (1990). CD44 is the principal cell surface receptor for hyaluronate. *Cell*, *61*(7), 1303–13.

Bane, A. L., Beck, J. C., Bleiweiss, I., Buys, S. S., Catalano, J. E., Daly, M. B., … O'Malley, F. P. (2007). BRCA2 Mutation-associated Breast Cancers Exhibit a Distinguishing Phenotype Based on Morphology and Molecular Profiles From Tissue Microarrays. *American Journal of Surgical Pathology*, *31*(1), 121–128.

Bane, A. L., Whelan, T. J., Pond, G. R., Parpia, S., Gohla, G., Fyles, a W., … Levine, M. N. (2014). Tumor factors predictive of response to hypofractionated radiotherapy in a randomized trial following breast conserving therapy. *Annals of Oncology 25*(5), 992–8.

Bane, A., Viloria-Petit, A., Pinnaduwage, D., Mulligan, A. M., O'Malley, F. P., & Andrulis, I. L. (2013). Clinical-pathologic significance of cancer stem cell marker expression in familial breast cancers. *Breast Cancer Research and Treatment*, *140*(1), 195–205.

Bapat, S. A., Koppikar, C. B., & Kurrey, N. K. (2005). Stem and Progenitor-Like Cells Contribute to the Aggressive Behavior of Human Epithelial Ovarian Cancer Stem and Progenitor-Like Cells Contribute to the Aggressive Behavior of Human Epithelial Ovarian Cancer. *Cancer Research*, *65*, 3025–3029.

Blohmer, J. U., Rezai, M., Kümmel, S., Kühn, T., Warm, M., Friedrichs, K., … Eiermann, W. (2013). Using the 21-gene assay to guide adjuvant chemotherapy decision-making in early-stage breast cancer: a cost-effectiveness evaluation in the German setting. *Journal of Medical Economics*, *16*(1), 30–40.

Boecker, W., & Buerger, H. (2003). Evidence of progenitor cells of glandular and myoepithelial cell lineages in the human adult female breast epithelium: a new progenitor (adult stem) cell concept. *Cell Proliferation*, *36 Suppl 1*, 73–84.

Bonnet, D., & Dick, J. E. (1997). Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nature Medicine*, *3*(7), 730–737.

Cancer Care Ontario. (2011). *Surgical Management of Early-Stage Invasive Breast Cancer Overview Guideline Report History*.

Cardoso, F., Harbeck, N., Fallowfield, L., Kyriakides, S., & Senkus, E. (2012). Locally recurrent or metastatic breast cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Annals of Oncology*, *23 Suppl 7*(Supplement 7), vii11–9.

Charafe-Jauffret, E., Ginestier, C., Bertucci, F., Cabaud, O., Wicinski, J., Finetti, P., … Birnbaum, D. (2013). ALDH1-positive cancer stem cells predict engraftment of primary breast tumors and are governed by a common stem cell program. *Cancer Research*, *73*(24), 7290–300.

Cheang, M. C. U., Chia, S. K., Voduc, D., Gao, D., Leung, S., Snider, J., … Nielsen, T. O. (2009). Ki67 index, HER2 status, and prognosis of patients with luminal B breast cancer. *Journal of the National Cancer Institute*, *101*(10), 736–50.

Clarke, M. F., Dick, J. E., Dirks, P. B., Eaves, C. J., Jamieson, C. H. M., Jones, D. L., … Wahl, G. M. (2006). Cancer stem cells--perspectives on current status and future directions: AACR Workshop on cancer stem cells. *Cancer Research*, *66*(19), 9339–44.

Clevers, H. (2011). The cancer stem cell: premises, promises and challenges. *Nature Medicine*, *17*(3), 313–319.

Collins, A. T., Berry, P. A., Hyde, C., Stower, M. J., & Maitland, N. J. (2005). Prospective identification of tumorigenic prostate cancer stem cells. *Cancer Research*, *65*(23), 10946–10951.

Creighton, C. J., Li, X., Landis, M., Dixon, J. M., Neumeister, V. M., Sjolund, A., … Chang, J. C. (2009). Residual breast cancers after conventional therapy display mesenchymal as well as tumor-initiating features. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(33), 13820–5.

Currie, M. J., Beardsley, B. E., Harris, G. C., Gunningham, S. P., Dachs, G. U., Dijkstra, B., … Robinson, B. a. (2013). Immunohistochemical analysis of cancer stem cell markers in invasive breast carcinoma and associated ductal carcinoma in situ: relationships with markers of tumor hypoxia and microvascularity. *Human Pathology*, *44*(3), 402–11.

Cuzick, J., Dowsett, M., Pineda, S., Wale, C., Salter, J., Quinn, E., … Forbes, J. F. (2011). Prognostic value of a combined estrogen receptor, progesterone receptor, Ki-67, and human epidermal growth factor receptor 2 immunohistochemical score and comparison with the Genomic Health recurrence score in early breast cancer. *Journal of Clinical Oncology*, *29*(32), 4273–8.

Dalerba, P., Cho, R. W., & Clarke, M. F. (2007). Cancer stem cells: models and concepts. *Annual Review of Medicine*, *58*, 267–84.

Dan, T., Hewitt, S. M., Ohri, N., Ly, D., Soule, B. P., Smith, S. L., … Simone, N. L. (2013). CD44 is prognostic for overall survival in the NCI randomized trial on breast conservation with 25 year follow-up. *Breast Cancer Research and Treatment*.

Dean, M., Fojo, T., & Bates, S. (2005). Tumour stem cells and drug resistance. *Nature Reviews*. *Cancer*, *5*(4), 275–84.

DeRose, Y. S., Wang, G., Lin, Y.-C., Bernard, P. S., Buys, S. S., Ebbert, M. T. W., … Welm, A. L. (2011). Tumor grafts derived from women with breast cancer authentically reflect tumor pathology, growth, metastasis and disease outcomes. *Nature Medicine*, *17*(11), 1514–20.

Dewan, M. Z., Ahmed, S., Iwasaki, Y., Ohba, K., Toi, M., & Yamamoto, N. (2006). Stromal cell-derived factor-1 and CXCR4 receptor interaction in tumor growth and metastasis of breast cancer. *Biomedicine & Pharmacotherapy*, *60*(6), 273–6.

Dontu, G., Abdallah, W. M., Foley, J. M., Jackson, K. W., Clarke, M. F., Kawamura, M. J., & Wicha, M. S. (2003). In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. *Genes & Development*, *17*(10), 1253–70.

Dos Santos, C. O., Rebbeck, C., Rozhkova, E., Valentine, A., Samuels, A., Kadiri, L. R., … Hannon, G. J. (2013). Molecular hierarchy of mammary differentiation yields refined markers of mammary stem cells. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(18), 7123–30.

Eirew, P., Kannan, N., Knapp, D. J. H. F., Vaillant, F., Emerman, J. T., Lindeman, G. J., … Eaves, C. J. (2012). Aldehyde dehydrogenase activity is a biomarker of primitive normal human mammary luminal cells. *Stem Cells*, *30*(2), 344–8.

Eirew, P., Stingl, J., Raouf, A., Turashvili, G., Aparicio, S., Emerman, J. T., & Eaves, C. J. (2008). A method for quantifying normal human mammary epithelial stem cells with in vivo regenerative ability. *Nature Medicine*, *14*(12), 1384–9.

Elston, C. W., & Ellis, I. O. (1991). Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. *Histopathology*, *19*(5), 403–410.

Ezeh, U. I., Turek, P. J., Reijo, R. a, & Clark, A. T. (2005). Human embryonic stem cell genes OCT4, NANOG, STELLAR, and GDF3 are expressed in both seminoma and breast carcinoma. *Cancer*, *104*(10), 2255–65.

Fang, D., Nguyen, T. K., Leishear, K., Finko, R., Kulp, A. N., Hotz, S., … Herlyn, M. (2005). A Tumorigenic Subpopulation with Stem Cell Properties in Melanomas. *Cancer Research*, *65*(20), 9328–9337.

Farmer, P., Bonnefoi, H., Becette, V., Tubiana-Hulin, M., Fumoleau, P., Larsimont, D., … Iggo, R. (2005). Identification of molecular apocrine breast tumours by microarray analysis. *Oncogene*, *24*(29), 4660–71.

Filipits, M., Nielsen, T. O., Rudas, M., Greil, R., Stöger, H., Jakesz, R., … Gnant, M. (2014). The PAM50 Risk-of-Recurrence Score Predicts Risk for Late Distant Recurrence after Endocrine Therapy in Postmenopausal Women with Endocrine-Responsive Early Breast Cancer. *Clinical Cancer Research*, *20*(5), 1298–305.

Filipits, M., Rudas, M., Jakesz, R., Dubsky, P., Fitzal, F., Singer, C. F., … Gnant, M. (2011). A new molecular predictor of distant recurrence in ER-positive, HER2-negative breast cancer adds independent information to conventional clinical risk factors. *Clinical Cancer Research*, *17*(18), 6012–20.

Fisher, B., Dignam, J., Wolmark, N., DeCillis, A., Emir, B., Wickerham, D. L., … Margolese, R. G. (1997). Tamoxifen and chemotherapy for lymph node-negative, estrogen receptor-positive breast cancer. *Journal of the National Cancer Institute*, *89*(22), 1673–1682.

Fogel, M., Friederichs, J., Zeller, Y., Husar, M., Smirnov, a, Roitman, L., … Sthoeger, Z. M. (1999). CD24 is a marker for human breast carcinoma. *Cancer Letters*, *143*(1), 87–94.

Geiss, G. K., Bumgarner, R. E., Birditt, B., Dahl, T., Dowidar, N., Dunaway, D. L., … Dimitrov, K. (2008). Direct multiplexed measurement of gene expression with color-coded probe pairs. *Nature Biotechnology*, *26*(3), 317–25.

Ginestier, C., Hur, M. H., Charafe-Jauffret, E., Monville, F., Dutcher, J., Brown, M., … Dontu, G. (2007). ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. *Cell Stem Cell*, *1*(5), 555–67.

Glinsky, G., Berezovska, O., & Glinskii, A. (2005). Microarray analysis identifies a death-from-cancer signature predicting therapy failure in patients with multiple types of cancer. *Journal of Clinical Investigation*, *115*(6), 1503–1521.

Greaves, M., & Maley, C. C. (2012). Clonal evolution in cancer. *Nature*, *481*(7381), 306–13.

Guo, D., Huang, J., & Gong, J. (2012). Bone morphogenetic protein 4 (BMP4) is required for migration and invasion of breast cancer. *Molecular and Cellular Biochemistry*, *363*(1-2), 179–90.

Harvey, J. M., Clark, G. M., Osborne, C. K., & Allred, D. C. (1999). Estrogen receptor status by immunohistochemistry is superior to the ligand-binding assay for predicting response to adjuvant endocrine therapy in breast cancer. *Journal of Clinical Oncology*, *17*(5), 1474–81.

Herschkowitz, J. I., Simin, K., Weigman, V. J., Mikaelian, I., Usary, J., Hu, Z., … Perou, C. M. (2007). Identification of conserved gene expression features between murine mammary carcinoma models and human breast tumors. *Genome Biology*, *8*(5), R76.

Horwitz, K. B., Dye, W. W., Harrell, J. C., Kabos, P., & Sartorius, C. a. (2008). Rare steroid receptor-negative basal-like tumorigenic cells in luminal subtype human breast cancer xenografts. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(15), 5774–9.

Hu, Y., & Smyth, G. K. (2009). ELDA: extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays. *Journal of Immunological Methods*, *347*(1-2), 70–8.

Jacobs, J. J. L., Kieboom, K., Marino, S., Depinho, R. A., & Lohuizen, M. Van. (1999). The oncogene and Polycomb- group gene bmi-1 regulates cell proliferation and senescence through the ink4a locus, *397*(January), 164–168.

Kondratyev, M., Kreso, A., Hallett, R. M., Girgis-Gabardo, A., Barcelon, M. E., Ilieva, D., … Hassell, J. A. (2012). Gamma-secretase inhibitors target tumor-initiating cells in a mouse model of ERBB2 breast cancer. *Oncogene*, *31*(1), 93–103.

Kristiansen, G., Winzer, K., Mayordomo, E., Kristiansen, G., Denkert, C., Dahl, E., … Dietel, M. (2003). CD24 Expression Is a New Prognostic Marker in Breast Cancer CD24 Expression Is a New Prognostic Marker in Breast Cancer. *Clinical Cancer Research*, *9*, 4906–4913.

Kurpios, N. A., MacNeil, L., Shepherd, T. G., Gludish, D. W., Giacomelli, A. O., & Hassell, J. A. (2009). The Pea3 Ets transcription factor regulates differentiation of multipotent progenitor cells during mammary gland development. *Developmental Biology*, *325*(1), 106–121.

Lahad, J., Mills, G., & Coombes, K. (2005). Stem cell-ness: a “magic marker” for cancer. *Journal of Clinical Investigation*, *115*(6), 1463–1467.

Leis, O., Eguiara, a, Lopez-Arribillaga, E., Alberdi, M. J., Hernandez-Garcia, S., Elorriaga, K., … Martin, a G. (2012). Sox2 expression in breast tumours and activation in breast cancer stem cells. *Oncogene*, *31*(11), 1354–65.

Lengerke, C., Fehm, T., Kurth, R., Neubauer, H., Scheble, V., Müller, F., … Staebler, A. (2011). Expression of the embryonic stem cell marker SOX2 in early-stage breast carcinoma. *BMC Cancer*, *11*(1), 42.

Li, X., Lewis, M. T., Huang, J., Gutierrez, C., Osborne, C. K., Wu, M. F., … Chang, J. C. (2008). Intrinsic resistance of tumorigenic breast cancer cells to chemotherapy. *Journal of the National Cancer Institute*, *100*(9), 672–679.

Lim, E., Vaillant, F., Wu, D., Forrest, N. C., Pal, B., Hart, A. H., … Lindeman, G. J. (2009). Aberrant luminal progenitors as the candidate target population for basal tumor development in BRCA1 mutation carriers. *Nature Medicine*, *15*(8), 907–13.

Liu, R., Wang, X., Chen, G. Y., Dalerba, P., Gurney, A., Hoey, T., … Clarke, M. F. (2007). The Prognostic Role of a Gene Signature from Tumorigenic Breast-Cancer Cells. *The New England Journal of Medicine*, *356*(3), 217–226.

Liu, S., Dontu, G., Mantle, I. D., Patel, S., Ahn, N., Jackson, K. W., … Wicha, M. S. (2006). Hedgehog signaling and Bmi-1 regulate self-renewal of normal and malignant human mammary stem cells. *Cancer Research*, *66*(12), 6063–71.

Mani, S. A., Guo, W., Liao, M. J., Eaton, E. N., Ayyanan, A., Zhou, A. Y., … Weinberg, R. A. (2008). The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell*, *133*(4), 704–715.

Marangoni, E., Vincent-Salomon, A., Auger, N., Degeorges, A., Assayag, F., de Cremoux, P., … Poupon, M.-F. (2007). A new model of patient tumor-derived breast cancer xenografts for preclinical assays. *Clinical Cancer Research*, *13*(13), 3989–98.

Marcato, P., Dean, C., Pan, D., & Araslanova, R. (2011). Aldehyde dehydrogenase activity of breast cancer stem cells is primarily due to isoform ALDH1A3 and its expression is predictive of metastasis. *Stem Cells*, *29*(1), 32–45.

Marjanovic, N. D., Weinberg, R. a, & Chaffer, C. L. (2013). Cell plasticity and heterogeneity in cancer. *Clinical Chemistry*, *59*(1), 168–79.

Mohsin, S. K., Weiss, H., Havighurst, T., Clark, G. M., Berardo, M., Roanh, L. D., … Allred, D. C. (2004). Progesterone receptor by immunohistochemistry and clinical outcome in breast cancer: a validation study. *Modern Pathology*, *17*(12), 1545–54.

Müller, a, Homey, B., Soto, H., Ge, N., Catron, D., Buchanan, M. E., … Zlotnik, a. (2001). Involvement of chemokine receptors in breast cancer metastasis. *Nature*, *410*(6824), 50–6.

Müller, B. M., Keil, E., Lehmann, A., Winzer, K.-J., Richter-Ehrenstein, C., Prinzler, J., … Denkert, C. (2013). The EndoPredict Gene-Expression Assay in Clinical Practice - Performance and Impact on Clinical Decisions. *PloS One*, *8*(6), e68252.

Muss, H. B., Woolf, S., Berry, D., Cirrincione, C., Weiss, R. B., Budman, D., … Norton, L. (2005). Adjuvant chemotherapy in older and younger women with lymph node-positive breast cancer. *JAMA : The Journal of the American Medical Association*, *293*(9), 1073–81.

Nagata, T., Shimada, Y., Sekine, S., Hori, R., Matsui, K., Okumura, T., … Tsukada, K. (2012). Prognostic significance of NANOG and KLF4 for breast cancer. *Breast Cancer*, *21*(1), 96 – 101.

Neve, R. M., Chin, K., Fridlyand, J., Yeh, J., Frederick, L., Fevr, T., … Berkeley, L. (2006). A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. *Cancer Cell*, *10*(6), 515–527.

Nielsen, T. O., Hsu, F. D., Jensen, K., Cheang, M., Karaca, G., Hu, Z., … Gilks, C. B. (2004). Immunohistochemical and Clinical Characterization of the Basal-Like Subtype of Invasive Breast Carcinoma Immunohistochemical and Clinical Characterization of the Basal- Like Subtype of Invasive Breast Carcinoma. *Clinical Cancer Research*, *10*, 5367–5374.

Nielsen, T., Wallden, B., Schaper, C., Ferree, S., Liu, S., Gao, D., … Storhoff, J. (2014). Analytical validation of the PAM50-based Prosigna Breast Cancer Prognostic Gene Signature Assay and nCounter Analysis System using Formalin-fixed paraffin-embedded breast tumor specimens. *BMC Cancer*, *14*(1), 177.

Paik, S., Shak, S., Tang, G., Kim, C., Baker, J., Cronin, M., … Wolmark, N. (2004). A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer. *The New England Journal of Medicine*, *351*(27), 2817–2826.

Paik, S., Shak, S., Tang, G., Kim, C., Baker, J., Cronin, M., … Wolmark, N. (2004). A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer. *The New England Journal of Medicine*, *351*(27), 2817–26.

Parker, J. S., Mullins, M., Cheang, M. C., Leung, S., Voduc, D., Vickery, T., … Bernard, P. S. (2009). Supervised risk predictor of breast cancer based on intrinsic subtypes. *Journal of Clinical Oncology*, *27*(8), 1160–1167.

Pastrana, E., Silva-Vargas, V., & Doetsch, F. (2011). Eyes wide open: a critical review of sphere-formation as an assay for stem cells. *Cell Stem Cell*, *8*(5), 486–98.

Perou, C. M., Sørlie, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S., Rees, C. a, … Botstein, D. (2000). Molecular portraits of human breast tumours. *Nature*, *406*(6797), 747–52.

Petrillo, L. a, Wolf, D. M., Kapoun, A. M., Wang, N. J., Barczak, A., Xiao, Y., … Esserman, L. J. (2012). Xenografts faithfully recapitulate breast cancer-specific gene expression patterns of parent primary breast tumors. *Breast Cancer Research and Treatment*, *135*(3), 913–22.

Prat, A., Parker, J. S., Karginova, O., Fan, C., Livasy, C., Herschkowitz, J. I., … Perou, C. M. (2010). Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. *Breast Cancer Research : BCR*, *12*(5), R68.

Prat, A., & Perou, C. M. (2009). Mammary development meets cancer genomics. *Nature Medicine*, *15*(8), 842–4.

Proia, D. a, & Kuperwasser, C. (2006). Reconstruction of human mammary tissues in a mouse model. *Nature Protocols*, *1*(1), 206–14.

Reynolds, B. a, & Weiss, S. (1992). Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science*, *255*(5052), 1707–10.

Ricardo, S., Vieira, A. F., Gerhard, R., Leitão, D., Pinto, R., Cameselle-Teijeiro, J. F., … Paredes, J. (2011). Breast cancer stem cell markers CD44, CD24 and ALDH1: expression distribution within intrinsic molecular subtype. *Journal of Clinical Pathology*, *64*(11), 937–46.

Ricci-Vitiani, L., Lombardi, D. G., Pilozzi, E., Biffoni, M., Todaro, M., Peschle, C., & De Maria, R. (2007). Identification and expansion of human colon-cancer-initiating cells. *Nature*, *445*(7123), 111–5.

Saghatchian, M., Mook, S., Pruneri, G., Viale, G., Glas, A. M., Guerin, S., … Van't Veer, L. (2013). Additional prognostic value of the 70-gene signature (MammaPrint®) among breast cancer patients with 4-9 positive lymph nodes. *Breast*, *22*(5), 682–690.

Shackleton, M., Quintana, E., Fearon, E. R., & Morrison, S. J. (2009). Heterogeneity in cancer: cancer stem cells versus clonal evolution. *Cell*, *138*(5), 822–9.

Shipitsin, M., Campbell, L. L., Argani, P., Weremowicz, S., Bloushtain-Qimron, N., Yao, J., … Polyak, K. (2007). Molecular definition of breast tumor heterogeneity. *Cancer Cell*, *11*(3), 259–73.

Shmelkov, S. V, Butler, J. M., Hooper, A. T., Hormigo, A., Kushner, J., Milde, T., … Rafii, S. (2008). CD133 expression is not restricted to metastatic colon cancer cells initiate tumors. *Journal of Clinical Investigation*, *118*(6), 2111–2120.

Shmelkov, S. V, St Clair, R., Lyden, D., & Rafii, S. (2005). AC133/CD133/Prominin-1. *The International Journal of Biochemistry & Cell Biology*, *37*(4), 715–9.

Singh, S., Hawkins, C., Clarke, I., & Squire, J. (2004). Identification of human brain tumour initiating cells. *Nature*, *432*(November), 396–400.

Singh, S. K., Clarke, I. D., Terasaki, M., Bonn, V. E., Hawkins, C., Squire, J., & Dirks, P. B. (2003). Identification of a Cancer Stem Cell in Human Brain Tumors Identification of a Cancer Stem Cell in Human Brain Tumors. *Cancer Research*, *63*, 5821–5828.

Sørlie, T., Perou, C. M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., … Børresen-Dale, a L. (2001). Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(19), 10869–74.

Sorlie, T., Tibshirani, R., Parker, J., Hastie, T., Marron, J. S., Nobel, A., … Botstein, D. (2003). Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(14), 8418–23.

Sugihara, E., & Saya, H. (2013). Complexity of cancer stem cells. *International Journal of Cancer*. *Journal International Du Cancer*, *132*(6), 1249–59.

Takaishi, S., Okumura, T., Tu, S., Wang, S. S. W., Shibata, W., Vigneshwaran, R., … Wang, T. C. (2009). Identification of gastric cancer stem cells using the cell surface marker CD44. *Stem Cells*, *27*(5), 1006–20.

Tirino, V., Desiderio, V., Paino, F., De Rosa, A., Papaccio, F., La Noce, M., … Papaccio, G. (2013). Cancer stem cells in solid tumors: an overview and new approaches for their isolation and characterization. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *27*(1), 13–24.

Tsang, J. Y. S., Huang, Y.-H., Luo, M.-H., Ni, Y.-B., Chan, S.-K., Lui, P. C. W., … Tse, G. M. (2012). Cancer stem cell markers are associated with adverse biomarker profiles and molecular subtypes of breast cancer. *Breast Cancer Research and Treatment*, *136*(2), 407–417.

van't Veer, L., Dai, H., van de Vijver, M. J., He, Y. D., Hart, A. A., Mao, M., … Friend, S. H. (2002). Gene expression profiling predicts clinical outcome of breast cancer. *Nature*, *415*(6871), 530–536.

Vermeulen, L., de Sousa e Melo, F., Richel, D. J., & Medema, J. P. (2012). The developing cancer stem-cell model: clinical challenges and opportunities. *The Lancet Oncology*, *13*(2), e83–9.

Visvader, J. E. (2011). Cells of origin in cancer. *Nature*, *469*(7330), 314–22.

Visvader, J. E., & Lindeman, G. J. (2008). Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. *Nature Reviews*. *Cancer*, *8*(10), 755–68.

Wicha, M. S., Liu, S., & Dontu, G. (2006). Cancer stem cells: an old idea--a paradigm shift. *Cancer Research*, *66*(4), 1883–90; discussion 1895–6.

Wolff, A. C., Hammond, M. E. H., Schwartz, J. N., Hagerty, K. L., Allred, D. C., Cote, R. J., … Hayes, D. F. (2007). American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. *Journal of Clinical Oncology*, *25*(1), 118–45.

Wright, M. H., Calcagno, A. M., Salcido, C. D., Carlson, M. D., Ambudkar, S. V, & Varticovski, L. (2008). Brca1 breast tumors contain distinct CD44+/CD24- and CD133+ cells with cancer stem cell characteristics. *Breast Cancer Research : BCR*, *10*(1), R10.

Yamanaka, S. (2008). Induction of pluripotent stem cells from mouse fibroblasts by. *Cell Proliferation*, *41*, 51–56.

Yu, F., Li, J., Chen, H., Fu, J., Ray, S., Huang, S., … Ai, W. (2011). Kruppel-like factor 4 (KLF4) is required for maintenance of breast cancer stem cells and for cell migration and invasion. *Oncogene*, *30*(18), 2161–72.

Zhang, X., Claerhout, S., Prat, A., Dobrolecki, L. E., Petrovic, I., Lai, Q., … Lewis, M. T. (2013). A renewable tissue resource of phenotypically stable, biologically and ethnically diverse, patient-derived human breast cancer xenograft models. *Cancer Research*, *73*(15), 4885–97.