Breast Cancer Stem Cells: Tumourspheres and Implications for Therapy

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Abstract

Breast cancer is a heterogenous disease, composed of tumour cells with differing gene expressions and phenotypes. Tumour heterogeneity has several important consequences for breast cancer including: (i) making classification by morphological and expression analysis more difficult because of the diversity within single tumours with the consequence that the majority of cells of the tumour will dominate this classification whether or not these cells are critical for diagnosis or treatment; (ii) treatments may fail to eradicate tumours simply by failing to eliminate one of the cell subtypes within the tumour; and (iii) differing abilities of the cell subtypes for dissemination and metastasis. Recently, a rare subpopulation of cells within tumours has been described with the ability to initiate and sustain tumour growth, to resist traditional therapies and to allow for secondary tumour dissemination. These cells are termed tumour-initiating cells or cancer stem cells, or alternatively, in the case of breast cancer, breast cancer stem cells. The therapeutic targeting of these cells has the potential to eliminate residual disease and may become an important component of a multi-modality treatment of cancer. Presented here is an investigation into: (i) ways to functionally and phenotypically identify breast cancer stem cells; (ii) the role of breast cancer stem cells in disease from both clinical samples and using xenograft assays; and (iii) the potential to target these cells.
Signed Statement of Originality

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

Signature: _______________________________ Date: ___________________________
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**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>Acute myeloid leukemia</td>
<td>AML</td>
</tr>
<tr>
<td>Aldehyde dehydrogenase-1</td>
<td>ALDH1</td>
</tr>
<tr>
<td>Allophycocyanin</td>
<td>APC</td>
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<tr>
<td>Blood brain barrier</td>
<td>BBB</td>
</tr>
<tr>
<td>Blood tumour barrier</td>
<td>BTB</td>
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<tr>
<td>Bone morphogenetic protein</td>
<td>BMP</td>
</tr>
<tr>
<td>Bovine serum albumen</td>
<td>BSA</td>
</tr>
<tr>
<td>Breast cancer stem cells</td>
<td>BCSC(s)</td>
</tr>
<tr>
<td>Cancer testis</td>
<td>CT</td>
</tr>
<tr>
<td>Cancer stem cell</td>
<td>CSC</td>
</tr>
<tr>
<td>Carboxy-fluorescein diacetate, succinimidyl ester</td>
<td>CFDA SE</td>
</tr>
<tr>
<td>Central nervous system</td>
<td>CNS</td>
</tr>
<tr>
<td>Checkpoint proteins</td>
<td>Chk</td>
</tr>
<tr>
<td>Cytokeratin</td>
<td>CK</td>
</tr>
<tr>
<td>Cytotoxic T-lymphocyte</td>
<td>CTL</td>
</tr>
<tr>
<td>Dendritic cells</td>
<td>DC(s)</td>
</tr>
<tr>
<td>Difference gel electrophoresis</td>
<td>DIGE</td>
</tr>
<tr>
<td>Double strand breaks</td>
<td>DSB</td>
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<tr>
<td>Dulbecco's Modified Eagle Medium</td>
<td>DMEM</td>
</tr>
<tr>
<td>Ethylenediaminetetraacetic acid</td>
<td>EDTA</td>
</tr>
<tr>
<td>Epidermal growth factor receptor</td>
<td>EGFR</td>
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<tr>
<td>Epithelial-mesenchymal transition</td>
<td>EMT</td>
</tr>
<tr>
<td>Ezrin-radixin-moesin-binding phosphoprotein 50</td>
<td>EBP50</td>
</tr>
<tr>
<td>(Basic) fibroblast growth factor receptor</td>
<td>bFGF</td>
</tr>
<tr>
<td>Fluorescein isothiocyanate</td>
<td>FITC</td>
</tr>
<tr>
<td>Fluorescence intensity</td>
<td>FI</td>
</tr>
<tr>
<td>Fluorescence-activated cell sorting</td>
<td>FACS</td>
</tr>
<tr>
<td>Fluorescent in situ hybridization</td>
<td>FISH</td>
</tr>
<tr>
<td>Foetal calf serum</td>
<td>FCS</td>
</tr>
<tr>
<td>Fold-expansion</td>
<td>F</td>
</tr>
<tr>
<td>Green fluorescent protein</td>
<td>GFP</td>
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<tr>
<td>Haematopoietic stem cell</td>
<td>HSC</td>
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<tr>
<td>Heat shock protein beta-1</td>
<td>Hsp27</td>
</tr>
<tr>
<td>Hedgehog</td>
<td>Hh</td>
</tr>
<tr>
<td>Haematoxylin and eosin stain</td>
<td>H&amp;E</td>
</tr>
<tr>
<td>Human mammary epithelial cell</td>
<td>HMEC</td>
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<tr>
<td>Humanised mammary fat pad</td>
<td>Hum. m.f.p.</td>
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<tr>
<td>Hydrocortisone</td>
<td>HC</td>
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<tr>
<td>Hypoxia-inducible factor</td>
<td>HIF</td>
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<tr>
<td>Immunohistochemical</td>
<td>IHC</td>
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<tr>
<td>Insulin</td>
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Invasive ductal carcinoma | IDC
Lineage | Lin
Long-term proliferating cell | LTP
Lymph node | LN
Magnetic resonance imaging | MRI
Mammary stem cells | MaSC(s)
Mean fluorescence intensity | MFI
Multi-drug resistance | MDR
N-acetyllactosamine | LacNAc
Neurosphere assay/medium | NSA
NOD.Cg-Rag1tm1MomIl2rgtm1Wjl/SzJ mice | NOD.Cg.Rag
Non-dividing cell | ND
Non-obese diabetic severe combined immunodeficiency | NOD/SCID
Oestrogen receptor | ER
Passage | P
Period Acid Schiff | PAS
Phosphate buffer saline | PBS
Phycoerythrin | PE
Polysaccharide modified citrus pectin | MCP
Progesterone receptor | PR
Propidium iodide | PI
Protein disulphide-isomerase | PDI
Reactive oxygen species | ROS
Recombinant human | rh
Retinoid acid | RA
Severe combined immunodeficiency | SCID
Short tandem repeat | STR
Short-term proliferating cell | STP
Side population | SP
Sodium dodecyl sulfate polyacrylamide gel electrophoresis | SDS-PAGE
Sphere forming capacity | SFC
Sphere forming efficiency | SFE
Subcutaneous | s.c.
Symmetric division rate of LTP cells | K_II
Terminal duct lobular units | TDLU
Tumour-associated antigens | TAA
Tumour-initiating cells | TIC(s)
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Chapter One: An Introduction to the Study of Breast Cancer Stem Cells

1.1 Introduction

The last twenty years have seen advances in the diagnosis and treatment of breast cancer. Despite this progress, breast cancer is still a leading cause of cancer related deaths among women, with as many as 40% relapsing with metastatic disease [1]. Breast cancer survival rates have been shown to plateau after 7-10 years, whereas most cancer survival curves take between 2 and 5 years to plateau [2]. The length of time for the survival rate to plateau in breast cancer might indicate the involvement of a cell type responsible for disease recurrence that is able to withstand primary treatment and reside in the body, often undetected, for prolonged periods. The clinical outcome of breast cancer is highly variable. Some breast cancer patients develop secondary tumours at distant sites following successful therapy of the primary tumour many years previously, while others presenting with disseminated disease at the time of diagnosis do not develop recurrences [3]. Interestingly, it has been shown that of the 40% of patients with lymph node involvement who did not undergo surgical removal, only 15% had recurrence of disease [4]. These findings raise the following points about breast cancer: (i) immune system surveillance of tumours or other protective mechanisms of the host might be capable of controlling some breast cancer relapses; and (ii) tumour dormancy exists in some breast cancers, allowing certain cells to reform a tumour. Identifying ways to recognise and eliminate those cells that have the potential to reform a tumour or metastasise will be critical for development of better therapeutics against breast cancer.

Prominent in the breast cancer field has been the notion of the existence of a transformed population of cells with many of the properties of stem cells that may be responsible for the origin and maintenance of tumours. These stem cell-like cells designated as breast cancer stem cells (BCSCs) or alternatively as tumour-initiating cells (TICs), represent a minor subset of cells in the tumour and are distinct from the more differentiated tumour cells. Understanding the biology of somatic mammary stem cells and BCSCs will be critical for investigating ways to target BCSCs. The stem cell compartment maintains tissue homeostasis in an organism throughout life. Stem cells are undifferentiated cells that have several basic defining properties that they share in common with TICs. One property is the ability to self-renew and give rise to multiple differentiated progeny (multipotency). In relation to cancer this property can partially
explain tumour heterogeneity. The relative quiescence of normal stem cells and TICs is another property they share in common and could explain the resistance of TICs to therapies aimed at mitotic cells. It is thought that cancer stem cells may play an important role in cancer establishment, progression and resistance to current treatments. Traditional cancer therapies are effective at debulking some tumours but often fail to produce long-term clinical remissions, possibly due to their inability to eradicate the cancer stem cell population. These cells are therefore an important target for novel treatments for both primary and metastatic tumours.

A variety of methods are being studied to disrupt the cancer stem cell pool, including inducing differentiation of the cancer stem cells or targeting of cancer stem cells for elimination. In particular, cancer stem cell antigens may provide a new target for cancer immunotherapy. The targeting of BCSCs through immunotherapy, such as dendritic cell (DC) based therapies or adoptive T-cell transfer, has the advantage of treating the putative cells of tumour origin and could augment current treatment regimes. This goal depends on finding antigens that distinguish cancer stem cells from differentiated cancer cells and somatic stem cells. This chapter will discuss stem cells and their role in mammopoiesis, cancer stem cells and their function in tumour formation, and the potential targeting of cancer stem cells for therapy with a focus on breast cancer.

1.2 Somatic stem cells

Somatic stem cells are responsible for tissue homeostasis in the adult, are capable of self-renewal, have limited plasticity, are responsible for tissue renewal and repair and can become activated in response to environmental signals such as hormones [5]. The slowly proliferating somatic stem cell generates a hierarchy of cells in the tissue that includes the more differentiated and faster proliferating transit-amplifying cell progeny, and the several lines of differentiated cells. Transit-amplifying cells can expand rapidly providing progeny that differentiate into mature cells of varying lineages. Non-proliferative differentiated cells make up the bulk of the tissue and undergo apoptosis after a finite life span. While stem cells are mostly quiescent, they may self-renew through asymmetrical cell division to give rise to one transit-amplifying cell and another stem cell [6]. This allows continuation of the stem cell compartment while providing the starting material for production of differentiated cells. Somatic stem cells may also divide symmetrically to produce two stem cells or two transit-amplifying cells and thus may either increase or decrease the stem cell pool. The plasticity of stem cells allows them, when activated, to renew several
lineages of differentiated cells during homeostasis. In adult tissue, the stem cell population rarely divides, but when stimulated by hormones during development or by a loss of transit-amplifying cells they can be rapidly activated to undergo asymmetric cell division to renew the tissue compartment. In this way, under most conditions, the total number of cells in a tissue is maintained in equilibrium, with the number of differentiated cells dying being equalled by the number of progenitor cells dividing. Understanding the role that the maintenance of cell division and differentiation of stem cells plays may lead to new insights into the signalling pathways involved in cancer progression. A prerequisite for such studies, and a major barrier for current research, is a set of accepted methods for stem cell isolation.

Stem cells have a relatively lineage negative (Lin⁻) phenotype and a few cell surface markers have been identified. Stem cells and progenitor cells are generally negative for cell surface protein markers that are used to define more differentiated lineage committed cells. As such, identification of other cell surface protein markers that are expressed by stem cells is a major focus of research. One property shared by normal stem cells and cancer stem cells is the expression of the ATP-binding cassette (ABC)-G2 transporter. The ABCG2 is a class of drug transporters capable of pumping out of the cell a variety of substrates including cytotoxic drugs, by utilizing ATP energy [7]. High expression of these transporters may help protect cancer stem cells from cytotoxic agents used for cancer treatment. The ABCG2 transporter has been shown to specifically pump out the fluorescent DNA-intercalating dye Hoechst 33342 [7]. Activity of this transporter leads to the identification of a population of cells known as the side population (SP) by flow cytometric analysis. This functional property has been used to study mammary stem cells, which upon transplantation into cleared mammary fat pads have been shown to give rise to breast tissue [8, 9]. However, other studies have called into question the use of the SP to isolate cells with potential to reconstitute a functional mammary gland [10]. Identification of other markers of mammary stem cells and BCSCs is an important avenue of research and will be discussed later.

1.3 The human breast and mammopoiesis

The development of the cellular lineages and functional units of the mammary gland is the process of mammopoiesis. These units are comprised of intralobular ductules and clusters of alveoli, which together form the terminal duct lobular units (TDLU). Collectively, TDLU form the branches of a greater ductal-lobular system composed of an inner layer of polarised luminal
cells and an outer layer of myoepithelial cells [11]. The adult human breast is composed of 15-20 lobes each with multiple lobules, surrounded by adipose and connective tissue. Milk is synthesised in differentiated acini of the mammary gland and carried through the ducts to the nipple. Additionally, the breast has a system of lymphatic vessels, responsible for draining breast tissue leading to internal mammary lymph nodes and axillary regional lymph nodes. The human breast is a dynamic gland with tissue homeostasis occurring during early development, puberty, within menstrual cycles, during pregnancy and lactation, and eventual involution during menopause. Somatic mammary stem cells are active in forming and maintaining breast tissue. Furthermore, X-chromosome inactivation studies have shown that entire areas of the breast, in particular individual TDLU, are monoclonal in origin [12-14]. These areas are derived from one progenitor cell determined during early embryogenesis that has undergone random inactivation of one chromosome around day sixteen.

The breast originates from the invagination of the epidermis into the underlying mesenchymal tissue during the 10-24 week period of gestation. This process gives rise to epithelial ducts, which in turn give rise to rudimentary lactiferous ducts. Unlike a variety of other organs, the human breast continuously undergoes morphological and functional changes well into adulthood, with a secondary onset during puberty and culminating with the greatest differentiation occurring during pregnancy and lactation. Oestrogen hormonal stimulation during puberty drives the ductal elongation of the breast with stem cell activity found in the terminal end buds [15]. Prolactin and progesterone drive ductal branching and formation of acini leading to the formation of mature breast tissue [16]. The two major lineages of epithelial cells that are found in the breast are the outer contractile myoepithelial or basal cells, and the inner luminal epithelial cells, comprising ductal and alveolar subtypes. Figure 1.1 graphically represents the relative location of myoepithelial cells and luminal cells to each other and to somatic stem cells in ducts. Myoepithelial cells are specialised contractile cells that form a sheath around the ductal network of the breast and that reside between the luminal cells and the basement membrane. They are characterised by expression of common acute lymphoblastic leukaemia antigen (CALLA) or CD10 [17], CD49f (α6 integrin) [18], CD44v6 [19], alpha-smooth muscle actin [20], vimentin [21], cytokeratin (CK) 5 and CK14 amongst other markers [22]. Luminal epithelial cells are distinguished by their expression of MUC1 [23], CD24 [24], varied expression of ErbB2 [25], CD133 [26], epithelial surface antigen (ESA) also known as EpCAM [27] and CK7, CK8, CK18, and CK19 [22] as well as varied expression of oestrogen receptor (ER) and progesterone receptor (PR). During periods of pregnancy and lactation the breast goes through further rounds of
development with an increase in cell growth and formation from the luminal epithelial lineage of functional milk secreting alveoli. Following these periods, and again during menopause, there is an involution through apoptosis of the breast tissue [28]. Hence, mature cells of the breast are in a near constant state of turnover, being replaced through the action of regulated progenitor populations throughout the adult female life. The most primitive of these cells is the mammary stem cell (MaSC), the cell that is capable of self-renewal and differentiation to renew luminal and
Figure 1.1. The ducts of the developing mammary gland. The differentiated inner luminal epithelial cell layers and outer myoepithelial (basal) cell layers are established as the terminal end buds move through the fat pad. Cap cells at the tip of the terminal end buds are thought to be the stem cells. They can undergo symmetric self-renewal or asymmetrical self-renewal to generate transit cells of a myoepithelial lineage or luminal lineage (known as 'body cells'). The ductal lumen forms as cells either undergo apoptosis or differentiate into luminal cells.
myoepithelial cells. Human mammary epithelial cell (HMEC) progenitors are defined for their ability to generate a more limited number of progeny of a select lineage or lineages. MaSCs and mammary epithelial progenitors have recently been characterised for both murine and human cells.

1.4 Mammary epithelial stem cells and epithelial progenitors: relationship to breast cancer

Basal and luminal cells represent the terminal stages of differentiation in the hierarchy of the human breast epithelium. The MaSCs reside at the base of this hierarchy and retain the ability to renew both luminal and myoepithelial lineages. In the 1950s, Deome and colleagues performed one of the earliest demonstrations of the existence of adult mammary stem cells in mice [29]. They used a limiting-dilution assay showing that clonal precursor cells are capable of forming functional mammary outgrowths in cleared mammary fat pads, the progeny of which could be serially transplanted. This cleared-fat pad assay consists of clearing the endogenous epithelium of mice followed by transplantation of a limited number of cells, less than $2 \times 10^4$, into gland-free mammary fat pads. Subsequently, it was shown that the an entire functional mouse mammary gland may be comprised of the progeny from a single cell [30]. A single cell with stem cell-like features and the phenotype of CD49f$^+$CD29$^+$CD24$^{low}$ is capable of forming a complete and functional mammary gland upon transplantation into the cleared-fat pads of female mice [10, 31]. MaSCs can be found throughout the mouse mammary tree, but their greatest frequency is near terminal end buds, and they occur with lowest frequency in lactating alveoli [32]. Identification of a pluripotent, self-renewing population of MaSCs capable of giving rise to more differentiated cells in the mouse mammary gland has implied the existence of similar hierarchy in the human mammary gland.

Identification of human MaSCs and HMEC progenitors has been more difficult due to the lack of a suitable in vivo model. As a result of this, much effort has been focused at identifying and characterising MaSCs and HMEC progenitors in vitro [33]. However, recent progress has been made in investigating these cells in xenotransplantation models. HMEC progenitors have been investigated utilising an approach that clears the fat pad of highly immunodeficient mice followed by humanisation with human fibroblasts to create an environment conducive for HMECs growth [34]. An alternative method suspends dissociated HMECs with fibroblasts in collagen gels that are implanted under the kidney capsule of hormone-treated immunodeficient mice [35]. This
latter report identifies a distinct rare population with a basal-like phenotype (CD49f EpCAM\textsuperscript{neg/low}) as the cells with regenerative capability. Studies of HMEC progenitors from normal human mammary tissue that has been enzymatically digested and cultured as single cells at clonal densities has led to the identification of three progenitor types: a common or bipotent progenitor, luminal progenitor and myoepithelial progenitor [36, 37]. In this scheme, the common progenitor is the more direct daughter of the MaSC and is capable of giving rise to both the luminal and myoepithelial progenitors. It is characterised \textit{in vitro} by the ability to give rise to “mixed” phenotype cultures with both luminal and myoepithelial cells. These progenitors have a MUC1\textsuperscript{+}CD133 EpCAM\textsuperscript{+}CD49f\textsuperscript{-}CD10\textsuperscript{-}Thy-1\textsuperscript{+} phenotype [37, 38]. Luminal progenitors can be isolated at the same time as the common progenitor, give rise to luminal cells and are characterised by a MUC1\textsuperscript{+}CD133\textsuperscript{+}EpCAM\textsuperscript{+}CD49f\textsuperscript{-}CD10\textsuperscript{-}Thy-1\textsuperscript{+} phenotype [37, 38]. These luminal progenitors have also been characterised as CD24\textsuperscript{+}CD133\textsuperscript{+} and can be distinguished from mature luminal cells which are CD24\textsuperscript{-}CD133\textsuperscript{-}CD49\textsuperscript{-}EpCAM\textsuperscript{+} [39]. The third progenitor type is a myoepithelial-restricted progenitor that arises from an enriched population of the common progenitor after serial passage, and which only gives rise to cells with a basal-like phenotype [36].

The identification of mammary stem cells and epithelial progenitors in the breast has pushed forward research aimed at understanding the relationships between normal HMECs and their malignant counterparts. An important avenue of research is aimed at understanding this relationship in the context of tumour initiation. One way to phrase this question is; do tumour cells arise from normal adult mammary epithelial stem cells, transit-amplifying cells or differentiated cells? One such study investigating tumour origin in the context of the hierarchy of the HMECs has isolated three distinct epithelial subsets; mature luminal cell that were CD49f EpCAM\textsuperscript{-} with a high proportion of cells expressing ER and PR, luminal progenitors that were CD49f\textsuperscript{+}EpCAM\textsuperscript{-}, and MaSCs or bipotent progenitors that were CD49f\textsuperscript{+}EpCAM\textsuperscript{-} [39]. Lim \textit{et al.} then compared the mammary gene signature of these three populations and a fourth population of stromal cells to the expression profile of six distinct molecular subtypes of breast cancer. It was found that the luminal progenitor signature was most associated with basal-like breast cancer, the MaSC gene signature was most associated with claudin-low and ‘normal-like’ breast cancer, the mature luminal cells signature was most associated with the luminal A and B subtypes, and the stromal signature was most associated with claudin-low breast cancer (Figure 1.2). Interestingly, this study also showed a correlation between the luminal progenitor signature and the signature for \textit{BRCA1} mutation-associated breast tissue indicating a possible role for \textit{BRCA1}-associated and
basal-like breast cancer being the result of oncogenic events in the luminal progenitor population. Studies of this type raise the issue of investigating the relationship between the various normal cellular phenotypes of the breast and their potential malignant counterparts (in the context of BCSCs).

Breast cancer subtypes include at least two different cellular phenotypes, one reminiscent of basal lineages and the other of luminal lineages [40]. This has led to the idea that various breast cancer subtypes might arise via mutations in different compartments of stem cells [41, 42]. In situ observations have identified candidate cells with stem cell-like feature of various phenotypes. Some of these observations have identified candidate stem cells that are ER+. Indeed, ER+ stem cells have been identified as being important in adult mammary gland homeostasis [42]. However, ER− stem cells resident in the mammary tissue have also been identified and might represent the more primitive mammary stem cells. It has been recently shown that the murine mammary reconstituting cells are ER− and PR− [43]. Recent experiments have demonstrated a role for BRCA1 being involved in the differentiation of human ER− stem/progenitor cells into ER+ luminal epithelial cells [44]. The deletion of BRCA1 results in the prevention of the transition of ER− stem cells into ER+ progenitor cells. Heterozygous mutations in the BRCA1 gene predispose women to breast and ovarian cancer [45], with tumours often being of the basal-like phenotype characterised by lack of expression of ER, PR, and HER2. These studies suggests a model in which a block in BRCA1-mediated transition from stem cells to progenitor cells results in an increase in ER− stem cells that can then be the pool of target cells for further mutation events. However, greater than two thirds of breast cancer tumours are ER+ and the majority of these tumours are dependent on oestrogen for growth and thus can be treated with hormonal therapy [46]. A model of breast cancer origin has been proposed in which ER+ tumours are derived from ER+ stem cells or ER+ early or late progenitor cells and ER− tumours are derived from the more primitive ER− stem cells [47]. Other models have postulated that ER+ stem cells, which rarely divide and are also resistant to hormonal therapy, can generate ER+ short term transit amplifying cells that in turn give rise to ER+ differentiated cells. These models suggest that the diversity seen in tumour types among patients could be a direct result of transformation events occurring in different lineages of stem cells or progenitor cells. Further defining the hormone receptor status of BCSCs will have important implications on the treatment of disease, as hormone receptor status can dictate treatment options and is known to be an indicator of prognosis. Clearly, the role of stem cells and progenitor cells in tumourigenesis is still being uncovered. Figure 1.2
The proposed hierarchy of human mammary epithelial cells and their potential relationship with breast cancer subtypes. The mammary stem cell has a functional capacity to generate large numbers of differentiated progeny and to self-renew. The common progenitor or bipotent progenitor is identified by its ability to generate colonies containing both luminal and myoepithelial cells. Continued culture in vitro of the common progenitor results in a population of cells that are restricted in their ability to only generate cells with basal features – this is the myoepithelial progenitor [36]. The luminal progenitor expresses CD49f, EpCAM and MUC1 and is restricted to generating mature luminal cell progeny with differential expression of CD49f and ER. Recently, a proposed model [39] utilizing gene expression comparisons between these populations and subtypes of breast cancer has been put forth and has revealed similarities between specific subtypes and cells of the HMEC hierarchy. Of particular note is the proposed similarity between the luminal progenitor population and the basal-like and BRCA1 mutation-associated breast cancer subtypes.
1.5 Role of stem cells/tumour initiating cells in tumourigenesis

Several models have been put forth for the initiation of cancer. The clonal origin of tumours theory has been used to explain how tumours form, and postulates that cancer originates from a single mutation occurring in a few cells or a single cell that eventually leads to uncontrolled and unlimited proliferation of a population of clonally derived cells [48]. Genetic alterations continue to accumulate as the tumour progresses, leading to activation of proto-oncogenes into oncogenes and inactivation of various tumour suppressor genes and ultimately giving rise to sub-types of cells in the tumour that have acquired several traits such as the ability to evade apoptosis, self-sufficiency in growth signalling, tissue invasion and metastasis, and limitless potential to replicate [49]. This model further postulates that mutations in various cells would allow for selection of cells to have a survival advantage over others, leading them to proliferate to a greater extent, and allowing those cells to seed new tumours capable of further rounds of clonal expansion. Building upon this model, there are two different theories for how tumour heterogeneity develops - the stochastic and hierarchical models. The stochastic model postulates that all or most cells in a tumour have the potential to be tumorigenic, capable of forming a new tumour through proliferation [50]. The hierarchical model postulates that only a rare proportion of the cells in a tumour have this tumorigenic capacity and that the rest of the cells represent terminally differentiated cells that lack the ability to proliferate and initiate tumours. The hierarchical model is in line with the cancer stem cell hypothesis, in which the aberrant cancer stem cell is the cell responsible for tumour initiation and maintenance rather than the differentiated cells that make up the bulk of the tumour. Figure 1.3 illustrates the differences between the stochastic and hierarchical models.

The deregulation of signalling pathway mechanisms involved in self-renewal have been implicated in the formation of TICs [50]. It is important to note that stem cells or progenitor cells may be implicated in certain breast cancer subtypes and not others. However, stem cells and cancer cells do share a number of important characteristics. Stem cells and cancer cells both have the capacity for self-renewal and extensive proliferation. In the case of tumour cells this takes the form of self-sufficiency in growth signalling and uncontrolled cellular proliferation while for stem cells this is a tightly controlled process that occurs during embryogenesis, organogenesis, and maintenance and repair of adult tissues.
Figure 1.3. Stochastic and hierarchical models of tumour initiation and development. (a) The stochastic model postulates that tumour cells are heterogenous, but that most or all cells have the capacity to function as tumour-initiating cells, although this might be a rare occurrence. (b) The hierarchical model postulates that only a specific subset of cells (tumour stem cells) have the capacity for extensive proliferation to sustain the growth of a tumour. The hierarchical model is in line with the cancer stem cell hypothesis.

Both cell types are long-lived with active anti-apoptotic pathways and telomerase activity [51]. This feature makes stem cells more prone to accumulation of damaging mutations and genomic instability despite active DNA repair mechanisms. For tumours, there are often even higher risks of accumulating mutations, as defects in DNA repair mechanisms are often present. Both cell types have a resistance to environmental toxins, chemotherapeutic and radiation agents, often as a result of multi-drug resistance (MDR) via expression of ABC transporter proteins and selection by chemotherapy. Additionally, it should be noted that stem cells are thought to be relatively resistant to radiation because they are slow cycling (amongst other characteristics). It is thought that cells in a tumour that are able to withstand radiation might be cancer stem cells (discussed in more detail in chapter seven). Stem cells and cancer cells also share the characteristic of being mobile, leading to migration and homing for stem cells and potentially to metastatic disease for cancer stem cells [52]. Anchorage independence is one of the most important characteristics of transformed cells (including metastatic cells) and is a property of normal stem cells. These characteristics of stem cells that are common to cancer cells suggests that fewer or different steps might be involved for stem cells to transform into tumour-initiating cells in comparison to differentiated cells. It is thought that the unregulated self-renewal in the stem cell compartment can give rise to TICs and from there to tumour heterogeneity, although this does not rule out the possibility that malignant transformations may occur in transit-amplifying/progenitor cells or even differentiated cells, and that stem cell-like properties are then acquired by these cells.

An important characteristic of stem cells is the capacity for self-renewal and the regulation of the balance between self-renewal and differentiation. TICs share this property and the associated signalling pathways responsible include Notch, Wnt/β-catenin, and Hedgehog (Hh). The Notch family of transmembrane signalling proteins is expressed in stem cells and early progenitor cells and is involved in cell fate development [53]. Activation of Notch signalling by its ligand Jagged-1 has been shown to be involved in the maintenance of self-renewal and plasticity for hematopoietic stem cells [54, 55]. In relation to TICs, activated Notch 4 has been shown to suppress differentiation of breast epithelial cells [56] and promote the development of mammary tumours [57]. This might indicate a role in the constitutive activation of Notch 4 signalling in the transition from stem cell to TICs in some solid tumours. Additionally, expression of Notch family members has been found on mammospheres (a cell culture condition enriched for stem cells – discussed later) and Notch ligands are capable of affecting the self-renewal and differentiation capacity of healthy mammary cells, supporting a role for Notch in breast cancer development [58].
The Wnt pathway is involved in cell fate determination in many organs including the developing mammary gland. A pro-oncogenic role for β-catenin, a downstream target of Wnt signalling, has also been described [59]. Wnt signalling has been shown to play a role in haematopoietic self-renewal and experiments in transgenic mouse models have shown that activation of the Wnt signalling pathway in stem cells can lead to epithelial cancers [50, 60]. Over-expression of Wnt in mouse mammary glands can also lead to increased mammary tumour formation [61]. Taken together this indicates the possible involvement of Wnt signalling pathway members and β-catenin in the deregulation of stem cells into cancer stem cells.

The Hedgehog/Patched pathway plays a role in embryonic growth and cell fate determination during development. Hh signalling has specifically been shown to play a role in self-renewal of hematopoietic stem cells [62]. The PITCH membrane protein, product of the tumour suppressor gene Patched, is a receptor for the Hedgehog family of signalling molecules and has been implicated in a role in early embryonic tumorigenicity [63]. Activation of the hedgehog pathway has been found in a variety of cancers including breast, prostate, and lung cancer. Signalling pathways involved in TICs represent an important target for cancer therapy, as targeting the inappropriate signalling involved in self-renewal maintenance and tumour progression could represent a novel way to eliminate the cells responsible for maintaining the tumour.

The functional assignment of a subset of cancer cells as TICs in vitro relies on the capacity of the subset to self renew, and to generate a large number of differentiated progeny. However, the gold standard remains the sustained capacity for tumour initiation during serial transplantation of the putative TICs into immunocompromised mice such as NOD/SCID (non-obese diabetic/severe combined immunodeficiency) mice. Evidence for the existence of TICs responsible for the initiation and maintenance of cancer has recently been presented for several tumours. During the 1990s, studies of acute myeloid leukaemia (AML) were among the first to demonstrate that transformed human stem cell-like cells were capable of being the origin of tumours [64, 65]. It was demonstrated that cells with a stem cell-like phenotype of CD34+CD38− could give rise to tumours in immunodeficient mice while those cells without a stem cell-like phenotype (CD34+CD38+) could not [65]. Subsets of cells with different stem cell-like phenotypes were then found to preferentially give rise to transplantable tumours in the brain [66, 67] and the breast [68, 69]. Thus TICs with a stem cell-like phenotype are involved in the initiation of some leukaemias and solid tumours.
1.6 Breast cancer stem cells

One indication that stem cells might play a role in breast cancer comes from epidemiology data on breast cancer incidence following radiation exposure. Women exposed to radiation in their late adolescence, during a time of active breast remodelling, following the Hiroshima and Nagasaki atomic blasts had the highest susceptibility of breast cancer 20-30 years later compared to women exposed at other age groups [70]. This suggests that oncogenic exposure at a time of high stem cell activity could increase the risk of breast cancer and that adult mammary stem cells can accumulate genetic changes leading to transformation over several years with the eventual development of solid tumour. Thus, a model of tumour formation and development that takes into account the role of stem cells and progenitors in the breast epithelial hierarchy as primary targets for mutation should be incorporated into the view of how breast cancer initiation occurs. Figure 1.4 illustrates one way that this could occur – through field cancerization. In this model, a mutational transformation (that is clinically silent) occurring specifically in a stem cell could be passed on to daughter transit-amplifying cells through asymmetric division, and then along to the several differentiated cells that make up the breast. This would thus generate a potentially large number of cells that might be susceptible to secondary mutational events, which would ultimately lead to clinically apparent disease. Uncovering the phenotype and tumorigenicity of BCSC has been a major focus of investigation in the last years, and will be critical for designing therapies aimed at eliminating or differentiating cancer stem cells. This is made more difficult by the realisation that breast cancer is a heterogeneous disease presenting as luminal A and luminal B, basal-like, HER2+/−, progesterone receptor +/−, and oestrogen receptor+/−, each with a different clinical prognosis [71-74]. Indeed, it is possible that one single phenotype will not be applicable to all BCSCs. Models of carcinogenesis are contentious, but the role that cancer stem cells play in tumour formation is beginning to be further defined. Recently, Clarke et al showed that human cells with some characteristics of stem cells, identified on the basis of CD44+CD24−/lowLin− expression, could form tumours when as few as 100 cells were injected into NOD/SCID mice [68]. When 20,000 cells without this phenotype were used they were unable to form a tumour (Figure 1.5 shows an example mouse tumour from this seminal work). These experiments indicate that tumour initiation could be driven by rare breast cancer stem cell-like cells. Future experiments will be needed to specifically identify the origin of these tumour-initiating cells in the hierarchy of human breast epithelial cells.
Figure 1.4. Field cancerization, one model for how stem cells are involved in tumour formation (a) Stem cells generate transit amplifying cells that terminally differentiate into mature luminal and myoepithelial cells. (b) A mutagenic event occurring in a stem cell can result in a field of transit and terminally differentiated cells that all carry that mutation. (c) A large number of cells are created that are targets for secondary mutations (maroon cells). (d) Clinically apparent disease results when a cell(s) receives additional mutagenic events.
Figure 1.5

Seminal works characterising breast cancer stem cells. (i) Histological (a-b), xenotransplant (c), and morphological (d-e) data demonstrating that CD44+CD24-low/Lineage- cells are part of the tumourigenic population (i.e. a BCSC phenotype). (ii) Demonstration of mammosphere growth technique (a), generation of a mammosphere from a single cell (b), propagation (c), sphere forming efficiency (d), CD44+CD24-low phenotype (g) and phenotype of mammospheres (e-f).
1.7 Studying mammary stem cells, BCSC and sphere assays

Advances in cell culture approaches have been important in identifying and studying mammary stem cells. The study of mammary stem cells in vitro has been based upon work identifying neural stem cells through a cell culture assay known as the neurosphere assay, which makes use of serum-free medium supplemented with epidermal growth factor and basic fibroblast growth factor [75, 76]. Application of the neurosphere assay culture conditions have been used to identify undifferentiated human mammary stem cells grown in culture [33] known as “mammospheres” or “tumourspheres”, and to identify a candidate human BCSCs [69] (Figure 1.5 shows an example of mammospheres/tumourspheres). These culture systems have shown that mammospheres exhibit stem cell-like functional properties and a variety of phenotypic properties (discussed further in later chapters). In these conditions, most cells undergo anoikis whilst rare cells divide and generate spheroid structures – mammospheres/tumourspheres. These spheres are composed of a variety of cell types, and this is important to realise when conducting studies of cancer stem cells within spheres. An early report of the lack or loss of markers of differentiated epithelium in spheres [69] may have promoted the idea of a dedifferentiated state of the entire spheroid structure. This report also indicated that sphere-forming efficiency and therefore breast cancer stem cell frequency increases with serial passage in cell lines [77]. Taken together these lines of evidence might suggest that propagation and passage as tumourspheres markedly enriches for breast cancer stem cells; and that the study of whole spheres, using expression platforms for instance [78], could be exploited to inform us of new breast cancer stem cell markers. However, contradictory evidence exists as to the heterogeneity and differentiation within both normal breast mammospheres and tumourspheres [33, 79]. Additionally, the limited self-renewal capacity shown by an inability to serially passage normal human breast mammospheres beyond five passages [80] indicates that enrichment of stem cells using this model is not definitively achieved and that functional differentiation of cells occurs. Generally agreed upon, however, is the idea that the sphere assay model has utility as a method to identify, not enrich for, stem cell activity. Recently, this model assay has been used to assess stem cell symmetric division [81] demonstrating that this model can be used to evaluate the population of cells within a heterogenous sphere that display the properties of cancer stem cells.

The sphere assay has been extensively used to study the properties of stem cell-like cells in a variety of cell types. Sphere formation has been reported in cells from primary breast tumour [69, 79], metastases [82] and established cell lines [69, 77, 82-84] and these tumourspheres were
shown to be enriched for tumour-initiating cells using *in vivo* xenograft assays. As a result, the tumoursphere assay gained popularity as a measure and model for studying breast cancer stem cells phenotypic and functional properties *in vitro*. It has been used to identify potential breast cancer stem cell regulators through characterisation, overexpression, knockdown or antagonist studies. These studies have identified HER2 [85, 86], CD49f [77], PTEN [87], EpCAM [68, 83], aldehyde dehydrogenase-1 (ALDH-1) [84, 88], DLL1 and DNER as important cancer stem cell regulators [89]. Additional candidate stem cell markers are waiting to be identified. Table 1.1 lists some phenotypic characteristics of breast cancer stem cells. These identifications have been possible often through flow cytometric approaches to identify proteins of interest in cancer stem cells.

Flow cytometry has been important in demonstrating that SP cells are present in the human breast [8, 33]. The SP is further increased in mammosphere cultures compared to freshly isolated primary tissue samples [33]. Flow cytometry can also be used to purify phenotypic subsets for further study. An early study found that isolated CD44⁺CD24⁻ breast cancer cells were capable of self-renewal, extensive proliferation, and differentiation along several mammary lineages, and grew as non-adherent mammospheres in appropriate media [69]. Subsequently a variety of studies have explored CD44⁺CD24⁻ cells and have shown that they display increased resistance to radiation [90], have enhanced invasive characteristics necessary for metastasis [91], and that the presence of these cells may favour distant metastasis [92]. A study using eight established human breast cancer cell lines has demonstrated that CD44⁺CD24⁻EpCAM⁺ cells are slow-cycling and resistant to chemotherapy [83]. However, the heterogeneity of CD44/CD24 phenotypes in BCSC has recently been explored by other investigators questioning the reliance on CD44⁺CD24⁻ cells as the definitive phenotype of stem cell-like cells [93, 94]. The work by Honeth *et al.* examining 240 human breast tumours demonstrated that the CD44⁺CD24⁻ phenotype is associated with basal-like tumours, characterised as negative for HER2, oestrogen receptor, and progesterone receptor, and particularly that this phenotype was associated with BRCA1 hereditary breast cancer [94]. They showed that there is considerable heterogeneity both within and among tumours in the expression of CD44/CD24 indicating that tumorigenicity might not necessarily be restricted to CD44⁺CD24⁻ cells. They also did not find any association between CD44⁺CD24⁻ phenotype status and distant disease-free survival or factors such as tumour size, nodal status, or S-phase fraction that have been linked with clinical outcome.
An important marker of some breast cancers that has been used extensively in clinical practice as a marker of some types of breast cancer and as a drug target is HER2. The product of the HER2/neu (ErbB2) oncogene, a member of the epidermal growth factor receptor tyrosine kinase family, is overexpressed in some subtypes of breast cancer and is associated with a poorer prognosis [95, 96]. Recently, the role that HER2 might play in BCSCs has been investigated. It was found that that HER2 overexpression can increase the pool of stem/progenitor cells, that HER2 signalling is the driving force behind carcinogenesis and invasion, and that these effects could be specifically inhibited by trastuzumab treatment [85]. HER2 overexpression has been linked to the expression of another putative stem cell marker ALDH1 in a study examining 491 breast cancer patients [85, 97]. Further investigation into the expression of this marker for breast cancer stem cells is warranted.

1.8 Breast cancer and metastatic disease

Results from experiments utilising primary human cancer tissue have shown that not all cells in a tumour are equivalent, and a minority tumorigenic cell population exists in solid tumours of both the breast and the brain that represents around 1-2% of the total tumour burden, and these cells could be responsible for metastatic disease [68, 98]. It should be noted that the cells that are capable of metastasising might display a different phenotype than the original tumour initiating cells. Targeting cells that have the potential to metastasise will be an important application of the BCSC field as these are the cells that cause the majority of mortality from breast cancer.

Circulating tumour cells often can be detected in patients with both primary and metastatic disease and the presence of these cells is often associated with worse survival [99]. Metastasis is a non-random and organ specific process undertaken by certain cell types. In the case of breast cancer, metastatic disease often follows a pattern involving metastatic cells trafficking to
Table 1.1 Phenotypic and Signalling Pathway Characteristics of Breast Cancer Stem Cells.

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<thead>
<tr>
<th>Factor</th>
<th>Characteristics</th>
<th>References</th>
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<tr>
<td><strong>Cell surface markers that are expressed by putative breast cancer stem cells</strong></td>
<td></td>
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<tr>
<td>ABCG2, drug transporter</td>
<td>ATP-binding cassette drug transporters capable of pumping cytotoxic drugs out of the cell</td>
<td>7</td>
</tr>
<tr>
<td>CD44</td>
<td>Involved in cellular adhesion, motility and metastases</td>
<td>68, 69</td>
</tr>
<tr>
<td>CD49f (α6-integrin)</td>
<td>Basal and endothelial cell distribution, candidate stem cell marker</td>
<td>77</td>
</tr>
<tr>
<td>EpCAM / ESA</td>
<td>Epithelial surface antigen, a cell adhesion molecule expressed on mammary tissue and tumours</td>
<td>68, 83</td>
</tr>
<tr>
<td>ALDH1</td>
<td>Aldehyde dehydrogenase 1, plays a role in differentiation of stem cells, activity predicts poorer clinical outcomes</td>
<td>94, 88, 97</td>
</tr>
<tr>
<td>CXCR1</td>
<td>Chemokine receptor involved in metastasis, increased expression on ALDH1+ BCSCs</td>
<td>88</td>
</tr>
<tr>
<td>CXCR4</td>
<td>Chemokine receptor involved in metastasis, increased expression in mammospheres</td>
<td>58, 101</td>
</tr>
<tr>
<td>ER</td>
<td>Oestrogen Receptor, potentially expressed on breast cancer cells, mammary progenitors and breast cancer stem cells</td>
<td>42, 47</td>
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<table>
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<tr>
<th>Signalling pathways that play a role in cancer stem cells</th>
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<tr>
<td><strong>Delta/Notch pathway</strong></td>
<td>Pathway involved in cell fate development and expressed in stem cells and early progenitor cells</td>
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<tr>
<td>Notch-4</td>
<td>Plays a role in mammary development, overexpression shown to promote mammary tumours</td>
<td>53, 56, 58</td>
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<tr>
<td><strong>Wnt signalling pathway</strong></td>
<td>Pathway involved in stem cell self-renewal, over-expression can lead to epithelial and mammary tumours</td>
<td>50, 61</td>
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<tr>
<td>β-catenin</td>
<td>A downstream target of the Wnt pathway, a pro-oncogenic role has been described</td>
<td>61</td>
</tr>
<tr>
<td>PTEN</td>
<td>PTEN knockdown enriches for stem cells (mediated by activation of the Wnt/β-catenin pathway)</td>
<td>87</td>
</tr>
<tr>
<td><strong>Hedgehog/Patched pathway</strong></td>
<td>Pathway involved in embryonic growth and cell fate determination</td>
<td>63</td>
</tr>
<tr>
<td>PITCH</td>
<td>A receptor for the Hedgehog signalling family, has been connected to early embryonic tumourigenesis</td>
<td>63</td>
</tr>
<tr>
<td>Epidermal Growth Factor Receptor</td>
<td>EGFR signalling has been found to be upregulated in BCSC and may be required for mammosphere formation</td>
<td>94, 119</td>
</tr>
<tr>
<td>HER2</td>
<td>Signalling molecule, overexpression linked to increasing pool of stem/progenitor cells, expression on ALDH1+ BCSCs</td>
<td>85, 86</td>
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regional lymph nodes, and then to bone marrow, lung and liver and brain [100]. Interestingly, it has been shown that homing and migration pathways initially identified in haematopoietic/leukocyte cells might be involved in BCSC and metastatic disease. CXCR4, a chemokine receptor expressed by haematopoietic stem cells that binds CXCL12, has been shown to be increased by a factor of four in mammospheres, and to be expressed in both metastatic breast cancer cells and neuroblastomas [58, 100, 101]. Additionally, the organs that form the main target of breast cancer metastasis have the highest expression of the ligand CXCL12 [100]. This indicates the importance of both the BCSC metastatic ‘seed’ and the ‘soil’ of the organ of metastasis for this process to occur. The CXCR4/CXCL12 pathway could provide a new target to specifically neutralise the cells in tumours that are capable of forming new metastases before metastatic disease occurs. Recently, Charafe-Jauffret et al. have been shown that breast cancer cell lines maintain a distinct subpopulation of cells with the expression of CXCR1/IL-8Rα and that IL-8 increased sphere formation, indicating a possible role for these cells and this pathway in metastasis [102].

The cancer stem cell compartment is thought to be the reason for initiation of disease, resistance to treatment and occurrence of metastatic disease. Hence, the identification and targeting of any of these cancer stem cells compartments, without toxicity to normal stem cells, will be an important goal of therapy of breast cancer. This treatment will probably find most utility in the setting of targeting cells with a metastatic potential. However, disrupting the cancer stem cell compartment is made more difficult by the realisation that disruption of supporting cells around the stem cells, or differentiated tumour cells around cancer stem cells, could have deleterious effects by disrupting putative stem cell niches.

1.9 Stem cell niches

Stem cell niches are defined as locations in a tissue that specifically can support the existence of somatic stem cells. Niches allow the repopulation of the stem cells compartment from migrating stem cells or even from differentiated cells if the stem cell compartment is depleted [5, 103, 104]. It is possible that tumour therapy that disrupts the stem cell niche through ablation of the surrounding differentiated cells could lead to the subsequent death of the cancer stem cells. Alternatively, tumour therapy that depletes stem cells, but does not eradicate the stem cell niche, could lead to re-population of the stem cell niche with additional cancer stem cells. Murine mammary stem cells have been shown to be resident in the peripheral caps of terminal end buds
Identifying candidate stem cell niches in human breast tissue has been difficult. In humans, terminal end buds are not as prominent structures and identifying stem cell zones has had to rely on microdissection followed by cell sorting and functional characterisation of putative cells to determine stem cell niches in ducts and lobules. Villadsen et al. recently identified a stem cell niche in the ductal tissue with cells with the characteristics of clonal growth, self-renewal, and bipotency and positive staining for putative stem cell markers K19 and K14 [106]. Identification of the properties of stem cell niches will be important for targeting BCSCs as it will be necessary to disrupt the inappropriate signalling that the stem cell niche may provide to achieve lasting clinical effects.

1.10 Breast cancer stem cells as therapeutic targets

The past two decades have seen over 30 new anti-cancer drugs being introduced; however, survival rates have only marginally improved for many forms of cancer [107]. In contrast to differentiated cancer cells, cancer stem cells are slow dividing, are resistant to apoptosis and a higher capacity for DNA repair making them more resistant to traditional methods of cancer treatment such as radiation and chemotherapy. In vitro results comparing differentiated breast cancer cells grown under monolayer conditions to CD24low CD44+ cancer stem cells grown using mammosphere conditions showed that the stem cell-like population was more resistant to radiation [90]. In addition stem cells express ATP-binding cassette drug transporters, which protect the cell from cytotoxic agents and may lead to multi-drug resistance. Current anti-cancer therapy is effective at debulking the tumour mass but treatment effects are transient with tumour relapse and metastatic disease often occurring as a result of the failure of targeting cancer stem cells. Figure 1.6 graphically illustrates potential differences between conventional therapies and therapies that target cancer stem cells. In order for therapy to be more effective, debulking of differentiated tumours must occur followed by targeting of the remaining surviving, often-quiescent, tumour stem cells. This could be accomplished by differentiating BCSC through differentiating therapy or eliminating them via immunotherapy or biotherapy [108].
Figure 1.6

Figure 1.6. Conventional therapies vs. therapies that target cancer stem cells. Therapies that target differentiated cells (making up the bulk of the tumour) but that leave cancer stem cells (CSC) intact might demonstrate tumour shrinkage in the short-term, but allow the tumour to grow back. Therapies that effectively kill the CSC pool render the tumour unable to maintain itself and the tumour ultimately degenerates.

1.11 Differentiation therapy targeting cancer stem cells

One way to target cancer stem cells is to induce the cancer stem cells to differentiate. Targeting the cancer stem cell pool to differentiate results in the loss of the ability for self-renewal - a hallmark of the cancer stem cell phenotype and the reason behind maintenance of the cancer stem cell pool. One differentiation agent used in the clinic is retinoid acid (RA) (vitamin A) [109]. RA and vitamin A analogues can promote differentiation of epithelial cells and reverse tumour progression through modulation of signal transduction. RA-based therapy followed by chemotherapy has found use in acute promyeloctyic leukaemia and could also find use in solid tumour therapy [110]. Recently, the use of bone morphogenetic protein (BMP)-4 has been described as a non-cytotoxic effector molecule capable of blocking the tumourigenic potential of human glioblastoma cells [111]. This therapeutic agent acts by reducing proliferation and inducing expression of neural differentiation markers in stem-like, tumour-initiating precursors. These findings are intriguing in light of the role that BMP-4 may play in some breast cancers [112]. Finding ways to specifically target BCSCs via differentiation therapy is an application that needs to be further explored.

1.12 Targeting stem cells for elimination

Much of cancer therapy research is focused on targeting specific markers on tumour cells that are over-expressed or mutated and that often represent essential genes/proteins or pathways thought to be important for the development of the cancer. For instance, traztuzamab (Herceptin®) targets the HER2/neu (ErbB2) oncogene, a member of the epidermal growth factor (EGFR) receptor kinase family, a protein over-expressed on roughly 30% of breast cancer tumours [113]. While these approaches have seen some clinical successes, the cancer stem cell model predicts that only by targeting the putative cancer stem cells will significant clinical remissions of the disease occur. It is important to note that tumours may not only be driven by mutated proteins and inappropriate signalling, but epigenetic mechanisms activating genes involved in ‘stem-ness’ such as Oct4, Nanog, and Sox2 could also contribute to tumour formation [114]. Reversal of these epigenetic switches of cancer stem cells could be one novel way to target cancer stem cells. New therapeutics aimed at eliminating cancer stem cells could also be achieved through a variety of methods: targeting the self-renewal signalling pathways critical for cancer stem cells, targeting the ABC drug transporters that cancer stem cells use to evade chemotherapy, or by inducing the
immune system to eliminate the cancer stem cells through various immunotherapeutic interventions. One recently identified group of anticancer compounds that might find use against BCSCs are mitocans, which cause apoptosis in cancer cells by destabilising mitochondria [115]. Therapies aimed at targeting signalling pathways have also been explored for targeting BCSCs.

1.13 Targeting of molecular signalling pathways and drug transporters

As noted in Table 1.1 a number of signalling pathways have been implicated in the self-renewal of BCSCs. As such, targeting these pathways may be an effective way to suppress breast cancer recurrence. The use of the steroid-like molecule cyclopamine to inhibit the Hedgehog signalling pathway has shown some promise in inhibiting the growth of medulloblastoma and could be used in treatments of other cancers [109]. The Wnt pathway can also be inhibited through a variety of mechanisms. Targeting of β-catenin has received a lot of attention as RA has been shown to inhibit β-catenin activity [116] and tyrosine kinase inhibitors such as imatinib (Gleevec®) have been shown to downregulate β-catenin signalling [117]. Finally, the Notch pathway has also been investigated as a target. An antibody capable of blocking Notch-4 has been used ex vivo to block the formation of mammospheres from primary human specimens [118]. This indicates the potential to block the self-renewal capacity of BCSCs in the patient with this antibody and opens up the use of other antibody therapies in the elimination of BCSCs.

In vitro experiments have shown the resistance of breast cancer stem cells to chemotherapy and radiation. Recent clinical evidence has established that tumorigenic breast cancer cells with high expression of CD44 and low expression of CD24 are resistant to chemotherapy [119]. Breast cancer patients receiving neoadjuvant chemotherapy had an increase in the CD44+/CD24low population of cells following treatment. These cells retained the capacity to form mammospheres (demonstrating self-renewal) and had an enhanced propensity for forming tumours in SCID/Beige mice compared to pre-treatment samples, increasing from 4 of 14 (29%) to 7 of 14 (50%) patient samples transferred. Treatment of patients with HER2 positive tumours with lapatinib, an EGFR and HER2/neu (ErbB-2) dual tyrosine kinase inhibitor, resulted in non-statistically significant decreases in the percentage of CD44+/CD24low population and in the ability for self-renewal as assessed by mammosphere formation. Thus inhibition of regulatory pathways involved in self-renewal may confer improved clinical outcomes by targeting breast cancer stem cells.

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The high expression of ABC transporters such as breast cancer resistance protein (BRCP-ABCG2) and multidrug resistance-associated protein 1 (ABCB1/MDRR1) is a property of stem cells that is also a feature of cancer stem cells [7]. These transporters provide a protective mechanism against xenobiotic toxins and are also partially responsible for the resistance of cancer stem cells to traditional therapies. Pheophorbide, a chlorophyll catabolite, is a specific probe for ABCG2 that causes inhibition of ABCG2 efflux properties [120]. The combined use of ABC transporter inhibitors and chemotherapy could be used to increase the efficiency of chemotherapeutic drugs to kill cancer stem cells [121]. ABC transporter inhibitors also cause inhibition of normal stem cells, leading to potential toxicity in the bone marrow, and play a role in the maintenance of the blood-brain barrier [122]. However, effective targeting of this molecule could be vital since it plays a significant role in the resistance of cancer stem cells to treatment.

1.14 Immunotherapy targeting breast cancer stem cells

Immunotherapy aimed at stimulating the immune system to recognise and eliminate tumour has been explored for many years, but has recently gained renewed interest. Many vaccines targeting solid tumours have been employed both pre-clinically and clinically in the treatment of cancer with varying success [123]. Interest in these vaccines has been bolstered by increased understanding of the role that the immune system plays in cancer and by the molecular identification of tumour-associated antigens (TAAs) that can be used as targets for therapy. In the case of breast cancer, evidence is now coming to light that the immune system is involved in the surveillance of cancer, is impaired by tumours during the progression of cancer, and can recognise and eliminate cancer. Dendritic cells (DCs) are central to these processes as a result of their role in innate immunity and in generating humoral and cellular immune responses. DCs are professional antigen presenting cells and initiators of adaptive immunity through processing antigens and presenting epitopes in the context of MHC to T-cells [124]. DCs are capable of stimulating cytolytic T-cell responses (CTL) to TAAs on tumours and are equipped with all of the necessary co-stimulatory and cytokine signals needed to drive an effective immune response to tumour. Figure 1.7 illustrates one way that immunotherapy could be used to target BCSCs.
Figure 1.7. Immunotherapy targeting breast cancer stem cells. Further characterisation of BCSCs will likely reveal expressed antigens that can be used to target them using immunotherapy or biotherapy approaches. This figure demonstrates one approach using dendritic cells and tumour-associated antigen (TAA) mRNA. Dendritic cells can process and present TAA to T cells to stimulate a cytotoxic T-lymphocyte (CTL) response, which is thought to be critical for tumour elimination. Importantly, this strategy could be used to specifically target BCSCs, perhaps after the bulk of the tumour has been removed using other treatments.
To induce long-lasting clinical responses using immunotherapy, targeting cancer stem cells may be required. To achieve this, specific antigens expressed on the cancer stem cells but ideally not by normal stem cells must be found and targeted. It is important to identify as many antigens as possible on BCSCs in order to develop a polyvalent vaccine approach, targeting several antigens. Therefore, cancer stem cells need to be further defined in terms of gene expression that determines stemness and identifying molecules that are involved in regulating stem cell qualities. Gene expression comparisons have been conducted and can be used to identify what genes are expressed by the cancer stem cell compartment compared to normal stem cells [125, 126]. Interestingly, recent work has demonstrated that the expression profile of breast cancer stem cells more closely resembles that of embryonic stem cells than that of adult stem cells. An embryonic stem cell-like gene expression pattern was found to be upregulated in the CD44+/CD24low tumourigenic fraction of cancer cells [127]. Additionally, mapping the transcriptional profile of embryonic stem cell-like genes in primary human breast cancer has revealed two classes of tumours: those with an embryonic stem cell-like activated program and those with an embryonic stem cell-like repressed program. Those tumours with an embryonic stem cell-like activated program were associated with poorer differentiated tumours that were more likely to progress to metastasis and death. As noted earlier, the CD44+/CD24low phenotype in human breast tumours has been found to be associated with basal-like tumours and particularly BRCA1 hereditary breast cancer, and has been linked to expression of CD49f, elevated expression of CK5/14 and EGFR, and low expression of ER, PR, and HER2 [94]. Basal-like tumours have often been linked to poorer prognosis. The occurrence of the CD44+/CD24low phenotype was found to be lower in tumours of luminal type and particularly HER2+ tumours, irrespective of ER status.

Several proteins could be potential targets of immunotherapy directed against BCSCs. Mammospheres express EpCAM, HER2, and CD49f among many other markers, which could potentially be used to identify or target BCSC [33]. The activity of aldehyde dehydrogenase-1 (ALDH1), a detoxifying enzyme that may play a role in the differentiation of stem cells, has been detected in both normal and malignant human mammary stem cells and can be used as a predictor for poor clinical outcomes [128]. High activity of ALDH1 identified the cells capable of self-renewal and high tumorigenicity in NOD/SCID xenografts. As previously mentioned, another molecule used to identify or target BCSCs is CD44, a membrane receptor involved in cell adhesion, motility and metastases and along with P-glycoprotein (the product of the MDR1 (ABCB1) gene of drug transporters), has been linked to MDR [129]. CD44 has been routinely used as a marker to purify and enrich breast cancer stem cells by selecting for cells that are
CD44+CD24−/lowLin− and EpCAM+ [68]. CD29 (β1-integrin) and CD49f expression has also been associated with murine mammary stem cells with a Lin−CD24+ phenotype [10, 31]. One report using the human breast cancer line MCF7 has shown that the SP phenotype can be used to identify cells with characteristics of cancer stem cells that express the tumour antigen MUC1, supporting a role for the SP and MUC1 in further analysis of human BCSCs [130]. A recent report has shown that BRCA1-deficient murine breast tumours contain heterogeneous cancer stem cell populations [131]. In this report, some tumours contained cells with a CD44+/CD24low phenotype, while cell lines derived from another tumour contained CD133+ cells, a phenotype associated with other cancer stem cells for brain, prostate and colon cancer [132], but has not been described in breast cancer. Importantly, both populations of cells expressed the stem cell associated genes Oct4, Notch1, Aldh1, Fgfr1, and Sox1. This study shows that although cancer stem cell populations may be heterogeneous, they in fact share a common set of characteristics such as expression of stem cell regulatory genes, expression of cell surface markers, mammosphere/tumoursphere formation and tumorigenicity in xenografts, which may be exploited for identifying and targeting cancer stem cells [133].

Understanding of the biology of BCSCs will help to determine the best way to target them. Determining what mutations or overexpressed proteins are present in cancer stem cells, how these aid either the stem cell-like phenotype or the tumourigenic phenotype of these cells, and how to best target these proteins is going to be a critical component of immunotherapy. This is made more difficult by the role that epigenetic regulation plays in cancer stem cells. Additionally, targeting universal TAAs such as telomerase reverse transcriptase (hTERT) and inhibitor of apoptosis proteins (IAP) might also be important for effectively targeting tumours with immunotherapy, as will combining these treatments with ones that target unique stem-ness-related antigens [134]. Several additional markers and signalling molecules, such as the Hedgehog/Patched pathway, as well as the ABCG2 drug transporters could be used as potential targets of therapy for breast cancer [122]. Additionally, markers that are used for migration of breast cancer stem cells such as chemokine receptors should also be explored as potential targets.

It is preferable that any therapy developed is able to target metastatic disease before metastases occur. Circulating tumour cells have been detected in the blood of patients with metastatic and primary tumours and has been linked with a decrease in survival times [99]. Recent evidence has indicated that metastatic spread can be an early event in tumourigenesis [3, 135, 136]. Gene expression studies have shown that the profile of the primary tumour of breast cancer patients can
be used to predict disease outcome, with a specific gene expression signature predictive of a short interval to metastatic disease [136]. The poor prognosis profile included genes involved in regulating cell cycle and angiogenesis. Studies such as this have challenged the traditional view of metastatic cells arising late in disease, and have stressed the importance of developing assays to identify disseminated disease early. The identification of molecular targets on disseminated tumour cells, targets that might also occur on BCSCs, could lead to better treatments. These treatments should preferably be applied during early stages of tumourigenesis before overt metastasis occurs. Immunotherapy is certainly one approach to targeting these cells as it has the potential to target even single cells for cell death, has the power to target systemic disease, and has the power to achieve durable clinical responses even for metastatic disease. Another critical component of immunotherapy targeting BCSCs is the determination of the number of BCSCs residing in the tumour and the ability to eradicate them with the treatment, as tumour regression will be dictated by any escape of BCSCs. Additionally, any immunotherapy approach will likely require additional therapies such as cytotoxic T-lymphocyte antigen (CTLA)-4 blocking of the T-cell regulation to overcome tolerance of the immune system to cancer [137]. Finally, the identification of appropriate antigens expressed on breast cancer stem cells needs to be conducted along with identifying the best way to stimulate an immune response utilising these antigens. While we now have a broader understanding of the genes and signalling pathways involved in stem cells and putative cancer stem cells, the application of immunotherapy targeting these molecules might not necessarily be useful since they are often expressed by healthy stem cells and indeed by a variety of other cells. Determining proteins expressed more exclusively by BCSCs will be beneficial for developing rational approaches for immunotherapy directed against these cells.

1.15 Cancer/testis and feto-oncoprotein antigens and cancer stem cells

Cancer/testis (CT) antigens are derived from proteins that appear to be expressed only in germ cells and tumours [138]. A range of tumours are capable of making hormones such as chorionic gonadotropin that are trophoblastic in origin [139]. A model for the trophoblastic origin of some tumours has been postulated in which tumours arise from germ cells that fail to reach the gonads/ovaries during development [140, 141]. CT antigens are currently being investigated for their role in tumour formation and as potential targets of tumour immunotherapy. CT antigens are ideal cancer antigens, because they are expressed by a proportion of cells within the tumour and are largely absent from healthy tissue other than the testis. Furthermore, immunogenic CT
antigens have been shown to be present in tumours such as melanomas and breast cancer [142] and have been used as the targets of tumour vaccines, such as MAGE-3A1 melanoma [143]. There are some intriguing shared characteristics between germ cells and tumour cells including; immortalisation, invasion, migration/metastasis, angiogenesis induction, and immune evasion through downregulation of MHC [141]. Immunohistochemical studies have shown that CT antigens are expressed on only a small proportion of cells in a tumour [144]. This small proportion of cells could represent the cancer stem cell compartment or the early progenitor cells. It has been postulated that CT antigens could serve as markers and potential therapeutic targets of cancer stem cells within tumours. Melanoma cell lines enriched for stem cells express various CT antigens, and some of these antigens are present on the majority of stem cells [145]. The relationship between cancer stem cells and the expression of CT antigens needs to be further defined and the exact role of CT antigens in both germ line and tumours remains a central question of research. A model for cancer arising from mutations in stem cells or early progenitor cells that gives rise to a phenotype of expression of CT antigens has now been postulated [141]. While the exact role of the CT antigens themselves might not be fully understood, the potential to target them through immunotherapy is exciting because they are expressed on a proportion of cells in a tumour, possibly the putative cancer stem cells, and they have been shown to be immunogenic.

1.16 Conclusions and aims of the thesis

The cancer stem cell hypothesis is a new paradigm that could have a major impact on the treatment of disease by suggesting a new target for cancer therapy. MaSCs need to be understood in the context of both mammary development and as potential sources of the BCSCs. Questions still remain about the exact relationship between normal HMECs and their potential various malignant counterparts. However, transformed MaSCs have been identified as a potential source of breast cancer, tumour relapse, and tumour metastases; as such, they have gained prominence as potential targets for immunotherapy of cancer. Current treatments of cancer have shown efficacy in removing the bulk of differentiated cancer cells, while failing to eliminate the cancer stem cells responsible for tumour relapse. Future therapies will need to effectively target the cancer stem cells to induce clinically significant remission of disease. Target antigens for breast cancer stem cells need to be further defined so that affective targeting of the BCSC compartment can be realised that spares normal stem cell niches but disrupts the cancer stem cell niche. New treatments will typically not be fully optimal by themselves and will need to be further developed
and placed into combination therapy with existing treatments. Therapies targeting BCSCs might be employed after debulking of the differentiated tumour tissue. This would allow immune surveillance to also more efficiently eliminate the few remaining cancer stem cells. Targeting BCSCs might be an attractive approach to treat breast cancer metastasis and relapse and could lead to significant increases in clinical remissions and quality of life for breast cancer patients when used in a multi-modal treatment regimen. The hypothesis of this thesis is that functional and phenotypic properties of BCSCs can be identified in tumourspheres derived from primary patient breast cancer samples and breast cancer cell lines. Overall aims of this project include: (i) identifying BCSC-like cells in primary tumours; (ii) an assessment of the sphere assay in cancer cell lines as a model for investigating functional (stem cell division, proliferation, sphere formation, radiation resistance) and phenotypic (morphological assessment of spheres, protein expression of cells within spheres) qualities of BCSCs; (iii) exploring ways to improve treatment of metastatic breast cancer disease with a cancer stem cell focus. It is especially relevant to accomplish these aims with the multiple different subtypes of breast cancer.

Future chapters of this thesis will explore the use of the mammosphere/tumoursphere model for investigating BCSCs. It is hypothesised that the tumoursphere model is a useful model for examining breast cancer stem cells within a heterogenous sphere. In particular, the establishment of in vitro tumoursphere culture from primary and metastatic breast cancer patient samples and a mouse model for tumour growth will be discussed in chapter three. Data relevant to demonstrating that the tumoursphere assay is a useful model for assessing cancer stem cell self-renewal and tumorigenicity will be discussed in chapter four. This thesis will also investigate assessing BCSC phenotype through methods such as fluorescence-activated cell sorting (FACS) and immunohistochemistry (chapter five) and protein discovery (chapter six) in order to elucidate new markers or validate previously identified markers to isolate or target BCSCs. Importantly, the work in this thesis investigates the cancer stem cell components from a variety of phenotypically and molecularly different breast cancer cell lines. Furthermore, this thesis will discuss the role of radiation and cell cycle in BCSCs (chapter seven) and touch upon uncovering treatments for metastatic breast cancer in chapter eight (an important issue that also relates to BCSCs). Overall, our data suggests that the tumoursphere model is a useful system for investigating breast cancer cells with some of the characteristics of stem cells.
Chapter Two: Materials and Methods

2.1 General materials and methods:

2.1.1 Neurosphere assay (NSA) media and passaging of tumourspheres. Cancer cell lines were grown as 'tumourspheres' by adapting the growth medium and methods used to culture neural stem cells in the neurosphere assay (NSA) [75]. All cells were grown at 37°C in 5% CO₂. NSA media consists of Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) (Gibco, Invitrogen Australia Pty Limited, Mount Waverley, VIC, Australia) containing 20 ng/mL rhEGF (R&D Systems, Minneapolis, MN, USA), 10 ng/mL rhbFGF (R&D Systems), 4 μg/mL heparin (Sigma, St. Louis, MO, USA), 10% human or mouse proliferation supplement (NeuroCult®, Stem Cell Technologies Inc., Vancouver, BC, Canada), 0.15% bovine serum albumin (BSA) (Sigma), and 1% penicillin G-streptomycin solution (Gibco). Spheres are routinely spun at a low speed during centrifugation, typically at 800 rpm for five minutes using a 5810R centrifuge (Eppendorf AG, Hamburg, Germany). Spheres are disassociated using 0.05% trypsin-EDTA (Gibco) for two minutes at 37°C. Cells are then pipetted to break up spheres further into single cells with the addition of equal volume trypsin inhibitor (10 mg recombinant bovine DNase 1 (Sigma), 0.14 g trypsin inhibitor from Glycine max (soybean), 16 mL HEPES (1M, Sigma), made up to 1 L MEM (Gibco), then filtered through a 22 μm filter (Millipore), aliquoted into 10 mL aliquots and stored at -20°C).

2.1.2 Determination of sphere forming efficiency. The sphere-forming efficiency (SFE) of cell lines at various passages (denoted as P1, P2, etc.) and after various treatments (trastuzumab treatment or radiation treatments for example) was assessed for various sets of experiments. This was accomplished by culturing a set number of single cells (5000, 3000, 1000, 500, 300, and/or 100) in 50 μL of NSA media in the wells of a 384 well optical bottom plate (Nunc Thermo Fisher Scientific, Rochester, NY, USA). Typically, 10-16 wells were used as replicates. After 5-7 days of culture the number of 3D floating multicellular spheroid clusters (not clumps of cells) formed was counted. SFE = (# of spheres formed / # of single cells plated) x 100.

2.1.3 STR profiling of cells lines. The authenticity of some cell lines was tested by comparing the short tandem repeat (STR) profile with published data. These cell lines include the
original MCF7 and BT-474 cells used for some experiments before purchase from ATCC of fresh cell lines, HBL-100, SUM-159-PT, MDA-MB-436, MDA-MB-157, and T47D. DNA from cell lines was obtained by pelleting cells and vortexing briefly in 1 mL of DNA lysis buffer, with 5 μl of proteinase K (5 mg/mL) (Roche Applied Science, Penzberg, Germany), followed by incubation at 65°C for two hours. 150 μl of 5M potassium acetate was then added and cells were mixed well and placed on ice for 10 minutes. The mixture was then split into two equal parts (roughly 550 μl each), placed into eppendorf tubes and 750 μl of chloroform was added. The mixture was vortexed and then spun at maximum speed in a desktop centrifuge for 15 minutes. The top layer of this mixture was then transferred to a new eppendorf tube and 750 μl of absolute ethanol was added. Samples were then inverted to mix and left at room temperature for 2 minutes. Tubes were subsequently spun at maximum speed using a desktop centrifuge for 5 minutes. Absolute ethanol was discarded and 70% ethanol (500 μl) was added. The sample was spun again at maximum speed for 30 seconds to 1 minute. Ethanol was discarded and the samples were allowed to air dry for 2 – 5 minutes. The DNA pellets were then resuspended in 100 μl of milliQ water. DNA concentration and purity was assessed using the Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). STR profiling was done by Lisa Bowdler (Molecular Epidemiology, Queensland Institute of Medical Research (QIMR)) as a paid service. Five markers included on the profiling kit (out of nine markers total) were listed on the ATCC website profile page for some of the cell lines – Amelogenin, D13S317, D5S818, D7S818 and vWA. The other markers included are CSF1PO, D16S539, THO1 and TPOX. These five markers were used to assess sample mix up or correct use of sample. Results indicated that MCF7, T47D and MDA-MB-436 matched their published genotype (Figure 2.1). HBL-100 STR profile is no longer available on the ATCC website as this line has now been identified as male in origin and no longer sold via ATCC. Results from the STR profile do indicate the presence of Y chromosome, suggesting that this cell line is HBL-100. SUM-159-PT STR profile is also not listed on ATCC and despite efforts to obtain the STR profile of this line, the identity of the line is not known. Results indicated that BT-474 and MDA-MB-175 were not the correct lines. As such, the data collected with those lines has been discarded, and new BT-474 cells have been purchased directly from ATCC and all future experiments have been conducted with those cell lines.

2.1.4 Mycoplasma testing and treatment of mycoplasma-contaminated cell cultures. Mycoplasma testing was routinely done throughout the course of these experiments. Mycoplasma testing was conducted using the MycoAlert assay control set (Lonza,
Figure 2.1

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Figure 2.1. Result of STR Profiling. STR profiling was conducted by Lisa Bowdler (Molecular Epidemiology, QIMR) as a paid service. Yellow indicates correct STR signature for the cell line and red indicates incorrect. Unfilled boxes indicate unknown.
Rockland, ME, USA). Briefly, one mL samples of medium from tissue culture flasks was collected and spun down to generate cleared supernatant. 25 μL of cleared supernatant was placed into a well of a white walled microplate. 25 μL of MycoAlert reagent was added to each sample and incubated at room temperature in the dark for 5 minutes. Luminescence (recorded as reading A) was then assessed using a luminometer (Perkin Elmer, TopCount NXT, Waltham, MA, USA). 25 μL of MycoAlert substrate was then added to the samples and incubated at room temperature in the dark for 10 minutes. Luminescence (recorded as reading B) was then assessed using a luminometer. Positive and negative controls were also run for each test (controls provided in kit). Ratios of reading B / reading A > 1.0 was indicative of being positive for mycoplasma contamination. In most cases, contaminated cell lines were discarded and earlier contamination-free stocks were used. In some cases, mycoplasma infection was treated with BM Cyclin (Roche Applied Science). Briefly, over the course of three to four passages cells were exposed to BM cyclin 1 anti-mycotic for 3 days and BM cyclin 2 anti-mycotic for 4 days. Cells were then tested again for mycoplasma-contamination at the end of treatment.

2.1.5 Flow-cytometric analysis for cell surface marker expression. The expression of various markers for both adherent conditions and mammosphere/tumoursphere conditions was analysed using fluorescent conjugated antibodies (BD unless otherwise noted) with the following specificities: EpCAM-APC and PE (clone EBA-1), CD44-PE (clone 515), CD44-APC-Cy7 (clone IM7) (Biolegend, San Diego, California, USA), CD24-FITC and -PE (clone ML5), HER2-PE (clone Neu 24.7), CD49f, and MUC1-FITC (clone HMPV). For some experiments galectin-3 was detected with Biolegend antibody PE-anti-mouse/human galectin-3 (Mac-1) (clone Gal397). The PE-mouse IgG2b, κ isotype control (clone MG2b-5; Biolegend) was used to indicate background staining in some experiments. Cells were washed twice in PBS 1% BSA before staining with antibodies. After staining (4°C for 20 minutes in the dark) cells were washed once and from 10,000 to 30,000 total events examined using either a FACSCanto or a FACSARia (Becton Dickinson, BD, San José, CA, USA). Data were analysed with FACSDiva (BD) software. Live cells were gated using either propidium iodide (Sigma) staining or Live/Dead Red® (Invitrogen). Live-gated cell data was analysed using FlowJo software (Tree Star, Inc., Ashland, OR, USA) to obtain information such as Δ-mean or Δ-median fluorescence intensity (FI) or positivity (%) of a particular marker above the baseline value for the unstained control. In this case, Δ refers to the parameter in question (mean, median, or positivity) with the background staining values removed. Fluorescent markers were measured using either linear or log scales as was deemed appropriate for the expression intensity of the markers measured.
Importantly, each technique gives equivalent results in comparison to matched samples measured on the same scale. Additionally, CD24/CD44 co-staining quadrant data was also acquired and analysed using conventional log scales.

2.1.6 Sphere size determination and photomicrographs. For sphere sizing in chapter 5 of this thesis, cells were seeded at $1 \times 10^6$ in low-adherent T75 culture flasks. 10 random fields of each flask of tumourspheres were digitally imaged 7 days after seeding using an Olympus (Olympus Corporation, Tokyo, Japan) digital camera on an Olympus CK40 light microscope. Sphere diameters were measured on their long-axis using Photoshop CS3. The average size of spheres in the 10 fields or greater than 100 spheres (whichever was greater) was calculated. Measurements were taken in two independent assays and representative results are shown in the figure. Sphere size determination reported in this thesis was conducted by Dr. Chanel Smart, University of Queensland, Centre for Clinical Research (UQCCR), from photos taken by both Chanel Smart and Brian Morrison. Analysis of measurements was done by Brian Morrison. Previous counts were conducted by Brian Morrison demonstrated similar results (data not shown). Additional photomicrographs used in this thesis were also taken using the Olympus digital camera.

2.1.7 Statistical analysis. Using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA) software, two-tailed unpaired or paired t tests with 95% confidence intervals were performed. For analysing trends, one-way analysis of variance was performed with a post test for linear trend. Spearman’s correlation tests were also performed. A Fisher’s exact test was performed to analyse the presence or absence of tumour in control and treated mice injected with MCF7 tumourspheres.

2.2 Chapter three materials and methods

2.2.1 Processing primary tumour samples. Specimens were procured with the informed consent of patients, under protocols approved by the relevant research ethics committees (Queensland Institute of Medical Research and Royal Brisbane and Women's Hospital). Tumour samples were received from the Royal Brisbane and Women’s Hospital pathology lab directly or through the Lakhani Lab (UQCCR and QIMR). Samples were stored in Leibovitz’s L15 media (Gibco) on ice prior to usage (typically between 30 minutes to 1.5 hours).
All samples received a patient sample ID, the letter Q followed by a number. Samples were first cut into fine pieces using cross-hatching technique with scalpels. Sometimes, whole tumour pieces from this step were frozen in dimethyl sulfoxide (Wak-chemie Medical GMBH, Steinbach/Ts., Germany)-10% foetal calf serum (FCS) (HyClone, Logan, UT, USA) or were used in experiments (tissue slice tissue culture or implantation into mice). When used in experiments these pieces were designated “whole tumour pieces.” To produce single cells suspensions further treatment in a mixture containing 1x collagenase/hyaluronidase (Stem Cell Technologies) was necessary. The only exception to that was for Q168, a breast cancer brain metastasis sample of a male patient. This sample was easily dissociated using mechanical means and 0.05% trypsin-EDTA (Gibco) alone. All samples receiving collagenase treatment were placed into a 1x collagenase containing mixture made up of either NSA media (experiments prior to August 2008) or Medium 199 (Gibco, Invitrogen) (experiments post August 2008). This change was due to the suggestion of members of the Max Wicha Lab, University of Michigan Comprehensive Cancer Center. Samples were incubated in a 37°C water bath for 1 hour to 2 hours with mechanical dissociation performed every 15 minutes by gently pipetting the mixture. Afterwards, cells were placed through a 40 μm cell strainer (BD Falcon, Bedford, MA, USA) to achieve single cell suspensions.

2.2.2 Adherent culture and tumoursphere culture conditions for primary tumour samples. A variety of culture methods were employed to grow cells. These include variations such as culturing whole tumour pieces after cross-hatching with the scalpel, culturing whole tumour pieces that did not get filtered after collagenase treatment, and culturing single cells after collagenase treatment and filtering. Media employed to culture cells in adherent conditions include, RPMI-1640 (Gibco) + 10% FCS, L15 (Gibco) media with varying concentrations of FCS, Opti-MEM (Gibco) media with varying concentrations of FCS and combinations of these mediums with NSA. For growing cells in tumoursphere-promoting media, NSA media was used as described elsewhere. All mediums received 1% penicillin G-streptomycin solution (Gibco). All cells were grown at 37°C in 5% CO₂. Cells were typically grown in T-25 tissue culture flasks (Nunc) at a concentration of 50,000 cells to 500,000 cells per flask in 5 mL volumes. Cells were assessed every 1-9 days for growth and for presence of spheres.
2.2.3 Xenograft model for primary tumour samples. A variety of strains of mice were used for this study. For tumorigenicity studies conducted in 2007, SCID/Beige mice housed in a colony at the University of Queensland Australian Institute for Bioengineering and Nanotechnology (AIBN) were used. For tumorigenicity studies conducted in 2009 before September, NOD/SCID mice (Animal Resource Centre, WA) were used [146]. Finally, for experiments conducted post September 2009, NOD.Cg-Rag1<sup>Rag</sup>/SjJ mice hereafter referred to as NOD.Cg.Rag mice (The Jackson Laboratory, Bar Harbor, Maine, USA) were used [146]. All animal research complied with the local animal ethics committee. For experiments conducted at QIMR this was done under ethics approval P1159. A variety of injection methods were also used. For experiments conducted in 2007, whole tumour pieces or single cell suspensions of cells without matrigel were injected subcutaneous (s.c.) into mice. For experiments conducted in post September 2009, single cell suspensions were injected within a 1:1 matrigel (BD, Franklin Lakes, New Jersey, USA) PBS solution in a total volume of 100 μl. Some animals received whole tumour pieces either s.c. or in the humanised cleared fat pad (Hum. m.f.p.). Additionally, some mice used in 2009-2010 also received before transplantation, 60-day release 17β-estradiol pellets (Innovative Research of America, Sarasota, Florida, USA) placed s.c. in the interscapular region. Mice were assessed weekly for any tumour growth.

2.2.4 Humanised mammary fat pad assay for primary tumour samples.

Three week old female mice were anaesthetised using a mixture of xylazine and ketamine (Parnell Laboratories, NSW, AUS). The number 4 inguinal mammary epithelium was removed as previously described [147]. Briefly, the mouse was placed ventral side up on a corkboard and taped down. The mouse was shaved and alcohol was spread over the inguinal area. The number 4 and number 5 nipples were located and a 1 – 1.5 cm midline incision was made beginning at the number 4 nipple. Angled lateral incisions were then made from the midline point between the two number 4 nipples and ending at a point between the number 4 and number 5 nipples so that the three cuts resemble an inverted Y. Using cotton tips and forceps the skin was then spread away from the body and subsequently pinned into the corkboard. A lymph node (LN) was located near the intersection of three veins. A triangular area defined by the blood vessel near the junction by the LN, the blood vessel on the fat pad bridge between the number 4 and 5 nipples, and the number 4 nipples was then cut away. A little amount of the fat pad was left intact to act as a “pocket” to receive the fibroblast cells. At this time, 2.5 x 10<sup>5</sup> unirradiated RMF/EG fibroblasts and 2.5 x 10<sup>5</sup> irradiated (4 Gy) fibroblasts were injected into the cleared fat pads 24 h after irradiation. RMF/EG cells are a line of GFP-labelled human telomerase-immortalized human
mammary stromal fibroblasts previously generated from a reduction mammoplasty, and were a
donate of Dr. Charlotte Kuperwasser (Whitehead Institute for Biomedical Research,
Cambridge, MA) [34]. These cells have been reported to be nontumourigenic (do not form
tumours in mice or form colonies in soft agar). The mouse skin was subsequently put back in
place and stapled using a 9 mm Autoclip system (BD). Staples were removed four to six days
later. Within two weeks of the humanisation of the fat pad, tumour cells are injected either as
single cells or as whole tumour pieces by carefully reopening the anaesthetised mouse and
injecting the cells or implanting them into the humanised fat pad.

2.3 Chapter four materials and methods

2.3.1 Propagation and culture of tumourspheres from established
breast cancer cell lines and sphere forming capacity assessment. The
adherent growth conditions of the cell lines used in this study are detailed in Chapter 4 Table 4.1.
The cell lines and other reagents were either obtained directly from the ATTC (Rockville, MD,
USA) or kindly donated by Chanel Smart, UQ, Centre for Clinical Research, AUS, Mike
McGuckin, Mater Medical Research Institute, AUS; Georgia Chenevix Trench, Queensland
Institute of Medical Research, AUS; Melissa Brown, School of Molecular and Microbial
Sciences, University of Queensland, AUS; Lily Huschtscha, Westmead, The University of
Sydney, AUS; Tony Blick, St. Vincent’s Hospital, Melbourne, Gillian Lehrbach, Garvan Institute
of Medical Research, Sydney, AUS. Cells were trypsinized from adherent culture with 0.05%
trypsin-EDTA (Gibco), quenched in normal growth media, washed twice in PBS and passed
through a 40 μm cell strainer (BD Falcon). The concentrations of single suspensions were
obtained using the Countess™ automated cell counter (Invitrogen) then seeded in sphere-
promoting culture in NSA media at a density of between 1 – 5 x 10^4 cells/mL in low-adherent 6-
well plates and 5 – 10 x 10^4 cells/mL in low adherent T-75 tissue culture flasks (Nunc Thermo
Fisher Scientific). All cells were grown at 37°C in 5% CO_2. Sphere forming capacity (SFC) as
reported in chapter five was assessed in cell lines by seeding 2–5 x 10^5 cells per well in triplicate
in low-adhesion 6 well tissue culture plates in sphere-promoting culture conditions and repeated
at least twice. The presence of spheres was assessed on a light microscope after 7 days in culture
and scored with the appearance of 3D multicellular structures greater than 40 μm in diameter.
SFC was classified as ‘-’ = no spheres observed, ‘+’ = <0.01% and ‘++’ > 0.01% proportion of
spheres observed from initial seeding amount of single cells. Assessment was done independently.
by Brian Morrison and Chanel Smart. Growth curves for cells grown under adherent conditions and tumoursphere conditions were conducted typically using T-25 culture flasks and with 250,000 single cells for the start of culture in triplicate (BT-474 cells were sometimes done in triplicate using a larger number of starting cells i.e. 1x10^6). Cells were passaged and counted every 5-7 days and cultured again at the starting frequency. These cell counts were used to assess the fold-expansion and symmetric division rate of the cell lines.

2.3.2 Established breast cancer cell lines xenotransplantation. Six-eight week old NOD.Cg.Rag mice (The Jackson Laboratory) were used for the s.c. injections of breast cancer cells. Before transplantation, the mice received 60-day release 17β-estradiol pellets (Innovative Research of America) placed s.c. in the interscapular region prior to cell injection [148]. Day 6 cultures of P2 tumourspheres were made into single cell suspensions as previously described (MCF7, KPL-1, and BT-474). Mice were injected s.c. on the right flank with 10^6 single cells resuspended in 100 μl of 1:1 matrigel (BD) PBS. Mice (n = 10) were followed every 1-3 days for assessment of tumour size. Ellipsoid volume of the tumours was measured using the formula (½ x length x width^2) and the endpoint of the experiments was determined when the tumour volume reached 520mm^3. Additional animal experiments were done in a similar manner as described here.

2.3.3. Cycling experiment for ZR-75-1 cells and tumorigenicity. Vybrant carboxy-fluorescein diacetate, succinimidyl ester (CFDA SE) cell tracer kit (Invitrogen) was used for experiments assessing slow-cycling cells. ZR-75-1 spheres were prepared as single cells as previously described for tumourspheres. Cells were washed twice in PBS. 1 μL of a 5 mM solution was added per 4 x 10^6 cells in 1 mL of NS media (adjusted up or down depending on cell numbers used – generally 1 to 4 x 10^6 cells used per experiment). Cells were mixed and left at RT for 10 minutes in the dark. The reaction was stopped by addition of 5-10 mL of ice cold NS media + 10% FCS. Cells were spun down and two additional washes in PBS were performed to remove FCS contamination. Cells were then placed in sphere-forming culture for 5-7 days. Cells were subsequently harvested and prepared as single cells. Cells were sorted using the (Beckman Coulter, Inc., Brea, CA, USA) into cells that were dividing fast (the bottom 15% of cells diluting out CFDA SE), medium cycling cells, and slowly cycling cells (the top 15% of cells expressing CFDA SE). After sorting, cells were washed, and injected into NOD.Cg.Rag^-/- mice as previously described at 500,000 cells per injection for fast and slow cycling cells, n=4 mice. On Day 21, tumours were harvested from all mice and using methods described in 2.2.1 single tumour cells
were generated. A second passage of these tumour cells from the fast and slow cycling tumours were implanted back into mice at 100,000 cells per injection, n=1 mouse, and tumour volume was assessed over a month.

2.4 Chapter five materials and methods

2.4.1 Immunohistochemistry phenotyping of spheres. Immunohistochemistry was performed by Chanel Smart, Patricia Keith, and Lynne Reid, UQCCR. Brian Morrison contributed to the design of the panel of markers to assess. After five days in culture, spheres were pelleted and fixed in 10% neutral buffered formalin for 1 hour before being processed for paraffin embedding. Four mm sections were cut for standard Haematoxylin & Eosin (H&E), Periodic Acid Schiff (PAS) stains or prepared for immunohistochemistry using antibodies and antigen retrieval methods described in Table 2.1. The following antigen retrieval methods were used: heat retrieval in a decloaking chamber (Biocare Medical, Concord, CA, USA) with 0.001M Tris/ethylenediaminetetraacetic acid (EDTA) pH 8.8, at 105°C for 15mins or 0.01M citric acid buffer pH 6.0, at 125 °C for 5 mins; 0.1% Chymotrypsin in 0.01M CaCl₂ + 0.05M Tris buffer, pH 7.8 at 37°C for 10 mins. Two detection kits were used: Dako EnVision+ (Dakocytomation, Carpinteria, California, USA) and Vectastain® Universal ABC kit (Vector laboratories, Inc. Burlingame, California, USA) according to the manufacturer’s instructions. Dako HercepTest™ kit was used for HER2 staining.

Table 2.1. Immunohistochemistry phenotyping method

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Supplier</th>
<th>Clone</th>
<th>Dilution</th>
<th>Antigen Retrieval</th>
<th>Detection Method</th>
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<tbody>
<tr>
<td>CD44</td>
<td>Dako</td>
<td>DF 1485</td>
<td>1:100</td>
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<td>Dako</td>
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<tr>
<td>CK19</td>
<td>Dako</td>
<td>BA17</td>
<td>1:40</td>
<td>Citrate</td>
<td>Vector</td>
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<tr>
<td>E-cadherin</td>
<td>Novocastra</td>
<td>36B5</td>
<td>1:100</td>
<td>Citrate</td>
<td>Dako</td>
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<tr>
<td>EGFR</td>
<td>Invitrogen</td>
<td>31G7</td>
<td>1:100</td>
<td>Chymotrypsin</td>
<td>Dako</td>
</tr>
<tr>
<td>EMA (MUC1)</td>
<td>Dako</td>
<td>Clone E29</td>
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<td>Vector</td>
</tr>
<tr>
<td>ER</td>
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<td>1:100</td>
<td>EDTA</td>
<td>Dako</td>
</tr>
<tr>
<td>ESA (EpCAM)</td>
<td>Novocastra</td>
<td>Clone VU-</td>
<td>1:30</td>
<td>EDTA</td>
<td>Dako</td>
</tr>
<tr>
<td>Her2</td>
<td>Dako</td>
<td></td>
<td></td>
<td></td>
<td>HercepTest™</td>
</tr>
<tr>
<td>Ki67</td>
<td>Dako</td>
<td>Clone MIB-1</td>
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<td>Vector</td>
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<tr>
<td>--------------</td>
<td>--------</td>
<td>-------------</td>
<td>-------</td>
<td>---------</td>
<td>--------</td>
</tr>
<tr>
<td>Laminin 1+2</td>
<td>Abcam</td>
<td></td>
<td>1:300</td>
<td>Chymotrypsin</td>
<td></td>
</tr>
<tr>
<td>Vimentin</td>
<td>Dako</td>
<td>V9</td>
<td>1:400</td>
<td></td>
<td>Vector</td>
</tr>
</tbody>
</table>

Sections were reviewed and described by a trained pathologist (Ana Cristina Vargas, UQCCR). At least 2 independent sphere preparations were observed for each antigen.

2.4.2 Cell sorting and analysis of MUC1\textsuperscript{hi} and MUC1\textsuperscript{lo} cells. Using the MUC1-FITC antibody and the MoFlo cell sorter (Beckman Coulter), the top 15% and bottom 15% of MUC1 expressing live-gated (propidium iodide) cells were sorted from adherent MCF7 or KPL-1 cells. Cells were immediately placed into tumoursphere-promoting conditions and assessed for SFE at P1 and MUC1 expression at P1 and P3. At times other cell populations were sorted (such as CFDA SE populations). These sorts were done in a similar manner as described here. Cell sorting was done at the QIMR Flow Cytometry Core Facility.

2.4.3 Trastuzumab treatment of cells. The levels of HER2 cell surface expression was assessed by flow cytometry for both adherent cells (T47D and MCF7) and P2 tumourspheres when treated with 20 μg/mL trastuzumab (Herceptin®, Genentech Inc., South San Francisco, CA, USA) compared to no treatment and 20 μg/mL rituximab (Genentech Inc.) treatment. This was accomplished by culturing adherent or sphere cultures through two successive passages in the presence of the antibodies or no treatment. These passages were spaced five days apart and antibody was refreshed once during each passage. Antibody concentration validation was conducted using a dilution of antibody (20, 40, 80 μg/mL) pulsed onto MCF7 and T47D cells adherent and sphere cells cultured in T25 flasks, and assessing the effect on cell surface expression of HER2 48 hours later. Results indicated that 20 μg/mL of antibody concentration was sufficient to knock-down expression of HER2 in this assay. Cell cycle, growth, and sphere forming efficiency was also assessed post-treatment with trastuzumab. Cell cycle was tested at 24 and 72 hours after initial treatment with trastuzumab in MCF7 cells, using the described methods for cell cycle analysis elsewhere in this manuscript. Sphere forming efficiency was tested using methods similar to previously describe. Briefly, 5000 or 1000 single cells were plated per well of a 384 well plate (n=10). Cells were plated in the presence of 20 μg/mL trastuzumab or rituximab or no treatment. Five days later sphere counts were assessed. Growth curves for adherent cells.
(BT-474, MCF7 and T47D) were also generated in the presence of trastuzumab or rituximab 20 μg/mL or without treatment for four passages for BT-474 and nine passages for MCF7 and T47D.

2.5 Chapter six materials and methods

2.5.1 Proteome determination. Samples used for comparing the proteome include P6 MCF7 tumourspheres and matched adherent cells, and P2 MCF7 tumourspheres and P8 tumourspheres. Tumourspheres and adherent cells are prepared as previously described. Matched samples were of the same time since last split as spheres – typically 6 days. Adherent cells were washed two times with PBS (15-35 mL) and then scraped off in a small amount of PBS using a sterile cell scraper. Samples were then washed an additional time in 15-35 mL PBS and transferred to 1.5 mL cryotubes (Nunc). Tumourspheres were harvest and washed three times with PBS (no trypsin treatment) before being transferred to 1.5 mL cryotubes. Cells were then snap frozen using a slurry of dry ice and absolute ethanol and stored at -80°C until use. Typically 2x10⁶ cells were used to generate 400 μg of protein. Proteome analysis was conducted in Prof. Jeffrey Gorman’s laboratory by Dr. Marcus Hastie utilising the GE Healthcare-Biosciences (Piscataway, NJ, USA) equipment for 2D separations, including an Ettan IPGPhor 3 isoelectric focusing unit and an Ettan DALT 6 electrophoresis tank. This equipment is coupled to a Bruker (Bruker BioSpin Pty Ltd, Alexandria, NSW, AUS) Proteineer gel image analysis system with DECODON Delta 2D software (DECODON GmbH, Greifswald, Germany) for analysis of the intensities of spots to obtain quantitative comparisons of proteins. Samples were compared by the 2D-IEF/SDS-PAGE-DIGE approach to identify candidate proteins of interest. These candidates were then isolated from Coomassie-stained 2D preparative gels and digested in-gel with trypsin. Digests were then fractionated by nanoHPLC and analysed by MALDI and electrospray ionisation mass spectrometry to identify peptides and the proteins from which they are derived. The instrumentation for protein mass spectrometry includes two Orbitrap-Fourier transform-mass spectrometers in, an ion-trap mass spectrometer with the recently invented electron transfer dissociation and a MALDI-TOF/TOF. Further analysis of data was done using Scaffold 3 software (Proteome Software, Inc., Portland, OR, USA) and Uniprot (http://www.uniprot.org/). An additional test was conducted with three biological replicates of MCF7 adherent cells, P2 and P5 tumourspheres following the same protocol as described above.
2.5.2 Test of N-acetyllactosamine (LacNAc) effect on tumourspheres and adherent cells. Phenotype, SFE, expression of galectin-3, viability, and changes in cell cycle of various cell lines was tested in the presence and absence of LacNAc (Sigma). LacNAc concentrations ranged from 0.03 mM to 6 mM. These experiments were conducted in 384 well plates or 24 well plates.

2.6 Chapter seven materials and methods

2.6.1 Irradiation of cells. Cells were platted at 250,000 cells per T-25 culture flask or 1 x 10^6 cells per T-75 culture flask in the appropriate medium (either as tumoursphere-promoting culture conditions or for adherent/monolayer conditions). Cells were placed into one of three groups for initial experiments modelling fractionated irradiation; control cells receiving no irradiation, 1 x 10 Gy cells receiving one 10 Gy exposure to irradiation on day 5, and 5 x 3 Gy cells receiving five daily doses of 3 Gy finishing on day 5. Cells were irradiated using a MDS Nordion GammaCell 40 (MDS Inc., Mississauga, Ontario, Canada). 24 hours after the last irradiation, some cells were placed into the sphere forming assay thus irradiated adherent cells became P1 spheres and irradiated P1 spheres became P2. Cells were cultured for 5 days in 384 well plates, 5000 or 1000 single cells plated per well, n = 16. After 5 days wells were analysed for number of spheres and photomicrographs were taken. Other cells were cultured for an additional 48 hours after the last irradiation (7 days culture total) and then analysed for cell cycle as previously described. Other cells for additional experiments received irradiation at 0, 2, 5, or 10 Gy on day 5 or day 6. Cells in these groups were analysed for cell cycle expression at 6 hours, 24 hours or 48 hours.

2.6.2 Cell cycle analysis by propidium iodide (PI) staining. Cells were prepared as single cells as previously described and a cell count was performed. Cells were then fixed in 70% ethanol solution on ice for 15 minutes. Cells were vortexed during the addition of ethanol to ensure that clumping did not occur. Cells were then pelleted at 800 rpm for 5 minutes and resuspended in 500 μl of PI solution for incubation at 37 °C 5% CO₂ for 30-40 minutes. PI solution is made in PBS and contains: 50 μg/mL PI (Sigma), 0.1 mg/mL RNase A (Roche) and 0.05% Triton X-100 (Sigma). After incubation 3 mL of PBS was added to the test tube and cells were pelleted at 1500 rpm for 5 minutes. Five-hundred μl of solution was left remaining in the test tube and cells were placed on ice ready for analysis. Analysis was performed using
FACSDiva software on the FACSCanto. Data was analysed using FlowJo software and the cell cycle analysis was completed using Watson Pragmatic modelling [149]. Cell cycle analysis by the FlowJo software has labelled G₀/G₁ cells as G₁ and G₂/M cells as G₂. As such I have kept this labelling convention throughout the thesis.

### 2.6.3 CFDA SE loading of cells and determination of slow-cycling cells.

Vybrant CFDA SE cell tracer kit (Invitrogen) was used for experiments assessing slow-cycling cells. Cells were prepared as single cells as previously described for either adherent cells or tumourspheres. Cells were washed twice in PBS. 1 μL of a 5 mM solution was added per 4 x 10⁶ cells in 1 mL of NS media (adjusted up or down depending on cell numbers used – generally 1 to 4 x 10⁶ cells used per experiment). Cells were mixed and left at RT for 10 minutes in the dark. The reaction was stopped by addition of 5-10 mL of ice cold NS media + 10% FCS. Cells were spun and for cells destined for culture as tumourspheres, two additional washes in PBS were performed to remove FCS contamination.

### 2.6.4 γH2AX and Caspase-3 staining of cells following irradiation.

AlexaFluor647-conjugated anti-H2A.X-phosphorylated (Ser139) (Biolegend) and PE-conjugated rabbit anti-active caspase-3 (BD Pharmingen) antibodies were used for these experiments. Upon double strand breaks of DNA, serine 139 of H2A.X becomes phosphorylated, and then called γH2AX. For γH2AX, staining was done according to protocol using around 100,000 cells per tube and a dilution of 1 μL of antibody to 30 μL of staining buffer (PBS - 1% BSA). After staining (4°C for 20 minutes in the dark) cells were washed once and examined with FACSDiva (BD) software from 10,000 to 30,000 total events collected. For caspase-3, cells were washed once in PBS and then fixed and permeabilised using Cytofix/Cytoperm kit (BD) for 20 minutes at room temperature. Cells were then pelleted and washed with Perm/Wash buffer (BD). Cells were then stained with anti-caspase-3 antibody using 20 μL of antibody per 1x10⁶ cells for 60 minutes at room temperature in the dark. Cells were washed once with Perm/Wash buffer and resuspended in Perm/Wash buffer and data was then collected as previously described.

### 2.7 Chapter eight materials and methods

#### 2.7.1 Cell lines.

Two cell lines were used for these experiments. MDA-MB-231 is a triple-negative basal-like breast cancer (ER-, PR-, non-amplified expression of HER2) cultured in
DMEM-10% foetal calf serum (FCS) [150]. MCF7 is a luminal breast cancer cell line (ER+, PR+, with expression but non-amplified expression of HER2) cultured in RPMI-10% FCS [150]. For culturing cells as tumourspheres this work has used the neurosphere assay based upon culturing neural stem cells in serum-free medium containing recombinant human epidermal growth factor (rhEGF) and basic fibroblast growth factor (rhbFGF) as previously discussed [75].

2.7.2 Intracerebral injections of MDA-MB-231 and MCF7 cells and treatment. SCID/Beige female mice (6 weeks old) were obtained from a colony housed by the University of Queensland Animal House and were maintained according to the University of Queensland Animal House guidelines. The research complied with national legislation and was authorized by the local animal ethical committee. Mice received 200,000 single cell injections of MDA-MB-231, MCF7 cells, or MCF7 tumoursphere-derived cells in 3 μL of PBS via intracerebral injection using a Hamilton syringe (Hamilton Company, Reno, NV, USA) directly into the right brain hemisphere sub ventricular zone (2 mm lateral from bregma, 3 mm deep) with the aid of a small animal stereotaxic system (ASI Instruments, Warren, MI, USA) (Figure 2.2a and 2c). Half of the mice in the MCF7 groups were treated with trastuzumab 30 mg/Kg delivered intraperitoneally. For mice receiving MCF7 and MCF7 tumoursphere-derived cells, on day 23 (n = 5) or day 36 (n = 4) mice were euthanised and perfused with 3.7% paraformaldehyde and brains were collected. Haematoxylin and eosin (H&E) stained brain sections were cut at 1 mm (with the aid of Dr Leonard da Silva, UQCCR) and scanned using a Scanscope XT® (Aperio Technologies, Vista, CA, USA). Tumour sizes were measured using ImageScope (Aperio Technologies) software. For MDA-MB-231 mice there were two groups: Group 1: animals treated with intraperitoneal injection of trastuzumab on the same day of the brain injections, n = 3; and Group 2: untreated controls, n = 3. Tumor development was monitored using magnetic resonance imaging (MRI) at weeks 4, 6 and 10. Animals in Groups 1 and 2 were sacrificed at week 10. MRI images were processed and the tumour sizes were measured by manual ROI segmentation using the software Paravision 4.0 (Bruker Biospin Pty. Ltd, Germany).

2.7.3 Magnetic resonance imaging of the mice. The tumour development was monitored using MRI at weeks 4, 6, and 10. MRI scans were performed with the aid of Dr Nyoman Kurniawan, University of Queensland. MRI scans were performed using Bruker 16.4T vertical wide-bore scanner (Bruker Biospin), equipped with a micro-mouse AHS probe and a head coil (20 mm SAW linear coil, M2M imaging) (Figure 2.2b). The animals were kept under
anesthesia with isoflurane (1% at flow rate of 0.5-0.7L/min), and respiration rate was monitored using BIOTRIG (University of Queensland), animal body temperature was maintained at 30° Celsius. The animal heads were scanned at 78 x 78 micron in-plane resolution and 1 mm slice thickness. The differentiation of the tumour and normal tissues were achieved using 2D T1-multislice spin echo sequence with TR/TE=600/9 ms, NEX = 8 and 2D diffusion-weighted imaging with TR/TE = 2400/21.6 ms, diffusion encoding was applied in the read direction, with the parameters d/D = 2/14ms, and b = 1200 s/mm2, with 50 kHz bandwidth. MRI images of week 10 control tumour and trastuzumab treated tumour were taken.
Figure 2.2. Materials and methods used in chapter eight. (a) Example of a mouse skull with representative landmarks: B, bregma; L, lambda; CS, coronal suture; SS, sagittal suture. (b) The 700 MHz magnetic resonance micro-imaging system used in these experiments in collaboration with Dr Nyoman Kurniawan, UQ. (c) Example of the small animal stereotaxic system used in these experiment.
Chapter Three: Tumourspheres Derived from Primary and Metastatic Cancer

3.1 Introduction

Breast cancer is the most common malignancy in women, accounting for 18% of all female cancers [151]. As such, development of models to investigate breast cancer for treatment or prevention is a major focus of scientific research. One way to achieve this goal is to establish in vitro models for cancer research in tissue culture and in vivo models in xenograft immunocompromised mouse assays. These models can then be used to scientifically test aspects of cancer growth, treatment or treatment resistance, or a variety of other experiments related to cancer. One important aspect of breast cancer research that has just recently been reported is the evidence for the existence of breast cancer stem cells [68, 69]. This evidence would not be possible without specific in vitro and in vivo mouse models of breast cancer. One specific in vitro model for breast cancer stem cell research has been the growth of cells under tumoursphere-promoting conditions, showing growth of undifferentiated cells capable of self-renewal and extensive proliferation as clonal non-adherent spherical clusters [69]. As mentioned previously, an important in vivo mouse model for breast cancer stem cell research has been to use flow cytometry to isolate specific subsets of cells (displaying CD44+CD24lowEpCAM+ cell surface phenotype) to investigate tumourigenic potential after transplantation into immunocompromised mice [68].

Established breast cancer cell lines have been widely used as in vitro models in cancer research. The majority of commonly used breast cancer cell lines (including MCF7, the most commonly used breast cancer cell line worldwide) are not derived from primary breast tumours but rather from pleural effusions or ascites fluid [152]. Coincidently, most breast cancer stem cell research has also made use of patient pleural effusion samples [68, 82, 97] or cell lines derived from pleural effusions/ascites fluid (MCF7, T47D, ZR75-1, SK-BR-3) [69, 77, 78, 83, 90, 130]. This means that most studies are only investigating the most aggressive tumour cells and not the primary lesion. This has the benefit of investigating cells that are likely to be more metastatic and hence important in late-stage disease and which might be enriched in tumour stem cells, but is also clearly not representative of the primary patient tumour. Investigating the biology of the primary patient tumour is of course important as: (i) it is clinically relevant as many drug
therapies are targeted against the primary tumour; and (ii) the primary tumour represents the initiation of disease and initiation is an important aspect of breast cancer stem cell research. As such, investigation into primary tumour samples should be an important component of breast cancer research.

Culture of primary breast cancer cell lines has long been known to be challenging, with one challenge being the overgrowth of epithelial cells with rapidly proliferating stromal fibroblasts [153]. Additional challenges faced by scientists in trying to establish breast cancer cell lines are reviewed in [152]. Problems associated with primary breast tumour culture include: (i) slow population doubling time and senescence or terminal differentiation after only a few passages (commonly two or three passages); (ii) determination that the established culture is truly tumourigenic as phenotypically there is little difference between cultured tumour cells and normal epithelium [154]; and (iii) cells isolated from human breast microenvironment may behave differently and lack important cell-cell interactions and cytokines present in the original tissue milieu. At least one report has investigated breast cancer stem cells grown as tumourspheres from primary patient samples [69]. Ponti et al. have reported the use of tumoursphere-promoting conditions to culture clonal populations of cells with some of the defining characteristics of stem cells (defining characteristics of stem cells are reviewed in [5]). Briefly, this report indicated that cells derived from primary tumour tissue grown as tumourspheres had functional properties of stem cells such as self-renewal, extensive proliferation, and differentiation along multiple lineages and phenotypic properties of stem cells such as the CD44+/CD24- cell surface phenotype. Thus, the in vitro model for culturing breast cancer stem cells is now established (tumoursphere model).

Since many primary tumours are hard to maintain in long-term immortalised culture, other culture methods have been employed to try to better recreate in vivo conditions. One of these methods involves use of a three-dimensional laminin-rich extracellular matrix system that allows for signalling from an extracellular matrix that is not possible in two-dimensional monolayer conditions [155-157]. It is possible that growth as tumourspheres also allows for better cell-cell interaction than monolayer culture conditions and may be considered another three-dimensional model system that does not make use of a defined extracellular matrix (discussed in chapter five). Another method is the tissue slice method in which 400 μm slices of tissue are cultured. This method has been used with prostate, breast and colon tumours and benefits from less harsh enzymatic and mechanical disassociation being used as well as the fact that normal tissue
architecture and cell-cell interactions are preserved [158]. A logical progression from these models is the implantation of tumour pieces that have only been mechanically disassociated into small blocks in a mouse model, i.e. an “in vivo incubator.” This model would allow cell-cell interaction to take place and might better conform to the original human tissue microenvironment, particularly if the tissue implantation takes place in a humanised mouse fat pad. After tumour growth has occurred, single cell suspensions of the tumour can be prepared and investigated in vitro or retransplanted into another mouse.

Establishing the capacity for growth of primary breast cancer (and other tumours) samples in tumoursphere-promoting conditions is an important aspect of research regarding cancer stem cells. The tumoursphere model is a method to functionally identify stem cell-like cells within a heterogenous sphere of breast cancer cells. It is also a method of growing cells in a three-dimensional format, and thus is thought to more realistically models in vivo conditions than monolayer culture. As such, this work endeavoured to establish primary breast cancer tumour samples and metastatic tumour samples in sphere-promoting conditions and in adherent culture from single cells or from blocks of tissue (tissue slice method). Where possible, it was endeavoured to investigate properties of tumourspheres derived from tumour samples that fit in with the current paradigm of breast cancer stem cells, such as self-renewal, long term proliferation, and CD44/CD24 phenotype. Finally, this work wanted to establish the practicality of the hypothesis that establishing mouse models of tumour growth as an “in vivo incubator” would allow for generation of additional cells from the starting tumour material for use in further experiments. Here, this thesis reports on the growth of primary breast cancer samples in tumoursphere-promoting conditions and in a xenograft model of breast cancer.
3.2 Results

3.2.1 Patient tumour samples can be grown in tumoursphere-promoting conditions. Twenty-one patient tumour samples were utilised for this study of in vitro growth, comprising 19 primary breast cancer samples of female patients, 1 male breast cancer brain metastasis, and 1 melanoma brain metastasis of unknown patient sex (Table 3.1). An additional female breast cancer patient sample was also grown under adherent conditions but never assessed for growth under tumoursphere-promoting conditions. This sample (Q151) was overgrown with fibroblast cells and subsequently was not used in this study. Out of the twenty-one samples, seven (33%) demonstrated some capacity for forming spheres as assessed microscopically between day 3 and 9 of culture. Four of these lines (19%) could be passaged two to three times and still generate spheres and one cell line (4.8%) could be passaged greater than three times in culture. Six of the seven lines that were able to form spheres were from female patients with primary breast cancer. Representative photomicrographs of spheres for Q184, Q186, Q189, Q190 and Q211 (an ER⁺ PR⁺ mixed ductal lobular tumour) are shown in Figure 3.1. In some cases sphere-like structures were noted to remain attached to the culture flask and “bubble-up.” These structures were noted in particular for Q184 grown in both tumoursphere-promoting conditions longer than 9 days (the time by which they should have been passaged) and in adherent conditions (Figure 3.2). The remaining cell lines that never formed spheres were cultured under adherent conditions before trying again to culture under tumoursphere-promoting conditions. This was never successful.
Table 3.1

<table>
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<th>Sample ID</th>
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<th>SFC</th>
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<td>NA</td>
<td>NA</td>
<td>NA</td>
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<td>-</td>
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<td>8.6.2007</td>
<td>-</td>
<td></td>
<td>No culture ever established.</td>
</tr>
<tr>
<td>Q168</td>
<td>13.7.2007</td>
<td>Male Breast Cancer Brain Met</td>
<td>+</td>
<td>Adherent cells and spheres cultured 3+ passages.</td>
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<tr>
<td>Q173</td>
<td>26.7.2007</td>
<td>-</td>
<td></td>
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<tr>
<td>Q176</td>
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<td>5.9.2007</td>
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<tr>
<td>Q218</td>
<td>23.11.2007</td>
<td>ER-PR-</td>
<td>-</td>
<td>No culture ever established.</td>
</tr>
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<td>29.11.2007</td>
<td>-</td>
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<td>ER-PR-</td>
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<td>-</td>
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<td>-</td>
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<tr>
<td>Q248</td>
<td>6.3.2008</td>
<td>ER+PR+</td>
<td>-</td>
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<tr>
<td>Q281</td>
<td>4.7.2008</td>
<td>ER-PR- IDC</td>
<td>+</td>
<td>Spheres could not be cultured past P2.</td>
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Table 3.1. Tumoursphere Growth Capacity of Tumour Samples. 19 primary tumours samples from female breast cancer patients were tested for their capacity for growth in tumoursphere-promoting conditions. Six of these were capable of sphere forming capacity (SFC) as marked by a ‘+’. Two brain metastases samples were also tested and one was found to be capable of sphere formation. Q151 was never tested for sphere forming capacity because of overgrowth of fibroblast cells. ER = Oestrogen receptor; PR = progesterone receptor; IDC = invasive ductal carcinoma.
Figure 3.1

Figure 3.1. Primary Tumourspheres. Twenty times magnification photomicrographs of (a) a representative day 7 (of passage 2 = day 26 total in culture) sphere of Q184, (b) day 7 sphere of Q186, (c) day 9 sphere of Q189, (d) day 9 sphere of Q190, and (e) day 7 sphere of Q211. Bar represents 100 μm.
Figure 3.2

(a) Cells grown in tumoursphere promoting conditions at 10x magnification and (b) the same sphere at 20x magnification. (c) Cells grown in adherent conditions at 10x magnification. (d) Twenty times magnification of adherent culture showing multiple fibroblast-like cells and some round undifferentiated cells. Bars represent 100 μm.

Figure 3.2. Attached Spheroid Structures for Q184. Photomicrographs of Q184 cells on day 14.
3.2.2 Breast cancer brain metastasis sample can be grown in tumoursphere-promoting conditions. Q168 was a breast cancer brain metastasis sample of male origin. This cell line generated a large number of spheres earlier in culture (Figure 3.3b). The cell line was also capable of passage beyond passage 3 in tumoursphere-promoting conditions (Figure 3.3a). However, later passages started losing numbers of cells while still retaining the capacity to form spheres. This is evident in the day 75 spheres that were still found for this cell line (Figure 3.3c). The matched adherent cells grown for this cell line were also capable of growth for three or more passages. This sample was easily dissociated mechanically and with trypsin and did not require collagenase treatment to generate a single cell suspension. The other brain metastasis sample, the melanoma brain metastasis sample (Q195), was tougher to cut using scalpels, and collagenase treatment was required to dissociate the sample into single cell suspensions. Culture in tumoursphere-promoting conditions did not produce spheres. Culture under adherent conditions did allow for culture and cells were noted to be visibly pigmented, indicating melanin content, and further indicating that the sample was a melanoma brain metastasis.

3.2.3 The CD44+CD24- phenotype is not enriched in tumoursphere-promoting conditions compared to adherent conditions. One cell line (Q281, an ER- PR- invasive ductal carcinoma (IDC)) that was capable of growth in tumoursphere-promoting conditions and adherent conditions (Figure 3.4c) was assessed on day 5 for CD24/CD44 phenotype. Cells were only sufficient for one test. The percentage of cells that were CD44+CD24- for adherent cells was 90% (with 7% of cells having a slight expression of CD24) (Figure 3.4a). The percentage of cells grown under tumoursphere conditions that were CD44+CD24- was 89% (with 8% of cells having a low expression of CD24) (Figure 3.4b). Furthermore, the Δ-median fluorescent intensity of CD44 was similar between the two conditions (3546 and 3592 for adherent and spheres respectively). This indicates that the CD44/CD24 phenotype of cells grown as spheres or adherent is similar in one case tested.
Figure 3.3

(a) Growth of Q168 as adherent culture (R10) and tumoursphere culture (NSA). Note, after P4 an attempt was made to culture cells in 24 well plate but eventually the cells stopped expanding. (b) Day 6, P1, Q168 cells grown in tumoursphere promoting culture at 10x and 20x magnification. (c) Day 75 sphere at 20x magnification. Bars represent 100 μm.
Figure 3.4. Primary Tumourspheres and CD44/CD24 Phenotype. CD44/CD24 expression for primary breast cancer sample Q281 (a) grown as adherent culture and (b) grown as tumoursphere culture. Δ-Median fluorescent intensity for CD44 for adherent was 3546 and for tumourspheres was 3592. Both cultures were low for expression of CD24. (c) Photomicrographs of day 5 Q281 cells grown in adherent and tumoursphere conditions at 20x ad 40x magnification. Bars represent 100 μm.
3.2.4 Primary tumours rarely grow in immunocompromised mice. Several samples were used to test the ability of either single cells or whole tumour pieces to be grown in immunocompromised animals. These include some samples previously tested in vitro (Q210, Q211, Q218, Q225, and Q227) and some samples that were never attempted to grow in vitro (Q204, Q410, Q423, Q468 and Q487). All of these samples represent primary breast cancer samples of female patient origin except for Q204, which was a prostate brain metastasis sample. A variety of methods were utilised for injection of cells, including using single cells or whole tumour pieces implanted s.c. or in the Hum. m.f.p. Additionally, a variety of mouse strains were used for these studies and some studies were conducted with oestrogen hormonal supplementation (summarised in Table 3.2). No tumour growth was observed under any of these conditions, except for Q468 tumour (an ER\(^-\) PR\(^-\) IDC). This tumour sample was able to grow when injected as whole tumour pieces (3 pieces) implanted into the cleared and humanised mammary fat pad of a six week old NOD.Cg.Rag mouse that also received hormonal supplementation. When 2.7\(\times\)10\(^6\) single cells were injected into a similarly prepared littermate no tumour was formed. Importantly, the contralateral fat pad of the same mouse was also humanised and no growth of the human fibroblast cells was noted, indicating that the tumour growth was from the implanted tissue (Figure 3.5b). This tumour had sustained growth occurring over a period of 125 days (Figure 3.5a). Upon removal from the animal, this tumour was cultured under adherent conditions (RPMI-10% FCS) and under tumoursphere-promoting conditions. Both conditions produced cells that were able to grow in tissue culture, with different phenotypes either as adherent cells or as tumourspheres (Figure 3.5c). Tumourspheres at passage one (day 5 of culture) were on average 67 ± 15 μm in size (range between 39 and 104 μm) as assessed by diameter of the long axis. SFE was found to be 0.35%.
<table>
<thead>
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</tr>
<tr>
<td>Q487 (ER+ PR+)</td>
<td>10.12.2009</td>
</tr>
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Table 3.2. Mouse experiments performed using human tumour samples. Unless otherwise noted, all samples are primary breast cancer samples. UNK = unknown number of cells; s.c. = subcutaneous; Hum. m.f.p. = humanised mammary fat pad. K = 1000 *See figure 5 for more information.
Figure 3.5. In Vivo Incubator for Tumour Growth. (a) Tumour volume for Q468 tumour. Whole tumour cells placed into the humanised mammary fat pad of a Nod.Cg.Rag−/− mouse, n = 1. Tumour size was first measured as a bump taken to be around 3mm x 3mm on day 30, this bump increased to twice its size by day 60, by day 75 the tumour was large enough to calculate the size using calipers. The mouse was euthanised on day 125. (b) Day 125, photograph of mouse with tumour (arrow), no tumour was noted on the contralateral side receiving only human fibroblast cells. (c) 4x photomicrographs of cells grown under tumoursphere-promoting and adherent culture conditions 5 days after removal from mouse and start of tissue culture. Black bars represent 100 μm.
3.3 Discussion

The culture of primary human breast cancer samples under tumoursphere-promoting conditions has not been widely reported. More reports have looked at the growth of human breast cancer samples in xenograft assays, which have identified phenotypes such as CD44+CD24-Lin− and ALDH1+ expression with tumorigenicity and cancer stem cell-like properties [68, 97]. These reports have made extensive use of pleural effusion samples. One report that did specifically look at the growth of breast cancer patient samples as tumourspheres utilised pleural effusion samples and concluded that tumoursphere culture of pleural effusion samples did enrich for tumourigenic cells [82]. This study sourced pleural effusion samples (some as old as 20 years) and determined that most samples tested (17/22) were capable of generating tumourspheres that could be passaged. However, the majority of these spheres were less than 50 μm in average diameter with seven of them being only 20 μm. This size is on the borderline of what could be defined as a sphere. Another report looked specifically at the ability of primary breast cancer samples to grow in tumoursphere-promoting conditions [69]. In 7 of 16 cases (3 of 8 oestrogen receptor–positive cancers, 2 of 6 oestrogen receptor–negative cancers, and 2 fibroadenomas), 10 to 15 days after plating tumourspheres were observed. These tumourspheres could be serially passaged, however, four of the seven primary cultures terminally differentiated within a few passages in vitro (i.e., four to eight passages), suggesting they did not contain long-term cells capable of self-renewal. Alternatively, three cell lines (oestrogen receptor positive ones) were capable of growth as spheres for more than 40 passages. Mostly primary breast cancer samples were used in this study as access to pleural effusions was not available. 6/19 primary breast cancer tumour samples tested for growth in tumoursphere-culture were capable of forming spheres. Spheres were typically between 50-120 μm in diameter. Of these lines four could be passaged four three cycles and one could be passaged more than three times. Serial passage of spheres indicates self-renewal and the ability for extensive proliferation – both hallmark characteristics of stem cells and cancer stem cells. The fact that most of our lines were not capable of sustained growth as spheres beyond two to three passages could indicate that short term proliferating cells might be largely responsible for growth of the tumoursphere in these cases. The one line that was capable of growth beyond three passages was derived from a breast cancer brain metastasis sample. Perhaps metastatic disease has a higher propensity for growth as tumourspheres. This is certainly evidenced by the high use of pleural effusion samples in the literature. This brain metastasis sample in fact was still capable
of self-renewal up to Day 75, perhaps indicating the presence of long-term proliferating cells. The oestrogen receptor status of all of our primary tumour samples was not known, so comparison to the results of Ponti et al. that only oestrogen receptor positive primary samples formed long-term tumoursphere culture is not possible. What is known is that both oestrogen receptor positive and negative primary tumours were capable of culture as tumourspheres in at least one case each in our hands.

As previously discussed, the CD44+CD24low/- cellular phenotype has been used extensively as the hallmark phenotype for breast cancer stem cells. Studies have shown that cells of this phenotype display increased resistance to radiation [90], have enhanced invasive characteristics necessary for metastasis [91], and that the presence of these cells may favour distant metastasis [92]. A study using eight established human breast cancer cell lines has demonstrated that CD44+CD24 EpCAM+ cells are slow-cycling and resistant to chemotherapy [83]. The CD44+CD24low/- population isolated from breast tumours and pleural effusions by Al-Hajj et al. have previously been shown to enrich for tumourigenic cells in a mouse model [68]. Sometimes, these samples were also gated for expression of EpCAM, suggesting that EpCAM positivity further increased tumorigenicity. Ponti et al. have previously reported that 95-96% of cells in mammospheres cultured from either cell lines or primary breast tumours do not express CD24; however, CD24/CD44 expression was not reported for uncultured primary cell isolates [69]. Grimshaw et al. have also reported on CD24/CD44 expression of pleural effusion samples that are capable of growth as tumourspheres. They have found expression of the CD44+CD24low/- phenotype ranged from as low as 0% to as high as 70.6% [82]. Interestingly, they have identified one sample that uniformly lacked expression of CD24 and CD44 and suggest that this phenotype might represent the true cancer stem cell phenotype. For these reasons we were interested in investigating the CD44/CD24 phenotype of our cell lines that were capable of forming tumourspheres and comparing this phenotype to the matched adherent cell cultures. Unfortunately, only one sample was available for this study with sufficient numbers of both adherent and tumourspheres for FACS analysis. When examined by FACS both the adherent cells and the tumourspheres expressed similar levels of CD44 and both were mostly negative for CD24. The finding that tumoursphere-promoting conditions did not enrich for the CD44+CD24low/- phenotype is probably indicative that this phenotype is not necessarily a marker for breast cancer stem cells. Recently, the heterogeneity of CD44/CD24 phenotypes in BCSC has been explored by other investigators questioning the reliance on CD44+CD24- cells as the definitive phenotype of stem cell-like cells [93, 94]. The work by Honeth et al. examining 240 human breast tumours demonstrated that the
CD44^+^CD24^- phenotype is associated with basal-like tumours - characterised as negative for HER2, oestrogen receptor, and progesterone receptor, and particularly that this phenotype was associated with BRCA1 hereditary breast cancer [94]. They showed that there is considerable heterogeneity both within and among tumours in the expression of CD44/CD24 indicating that tumorigenicity might not necessarily be restricted to CD44^+^CD24^- cells. This finding is in accord with our result indicating that tumoursphere conditions do not enrich for this particular phenotype and rather that both the “non-differentiated” tumourspheres and the matched adherent/differentiated cultures share the same phenotype.

Our finding that tumoursphere-promoting conditions could generate tumourspheres in primary samples but that these spheres were often not capable of sustained serial passage, prompted us to assess the ability of primary tumours to grow in immunocompromised mice. Indeed, this was always going to be one important aspect of this work, in order to demonstrate tumorigenicity of putative populations of cells, but was even more critical since cell lines were difficult to establish in tissue culture from primary patient samples. It was/is hoped that mice might act as an in vivo cell incubator, allowing for the enrichment of tumourigenic cells that could then be analysed ex vivo for properties of cancer stem cells. This phase of the research required a great degree of optimising, and indeed was aided by learning the cleared and humanised fat pad technique first hand in the laboratory of Dr. Max Wicha, University of Michigan Comprehensive Cancer Center. One aspect of optimisation involved the strain of mice used. This work started off using SCID mice (here meaning either SCID/Beige or NOD/SCID) as has previously been used in the literature [68]. We did not have success growing primary tumour samples (and one prostate brain metastasis sample) using SCID mice. It is possible/probable that the strain of mice being SCID was not the issue and that the tumours did not grow because of some other reason – i.e. lack of hormone stimulation, insufficient cell numbers, poorly prepared cell samples or some other reason not specific to the strain of mouse. Overall, nine samples were tested for growth in SCID mice, all using a subcutaneous route of injection and either as whole tumour cells or single cells with and without matrigel and with or without exogenous hormonal stimulation. In no case did tumours grow. As a result of this finding, coupled with the result that the NOD/SCID mice that we had sourced internally to QIMR displayed some level of T, B, and NK cells and did not allow for tumour take of known tumourigenic cell lines (data not shown), we switched to using NOD.Cg.Rag mice. This switch was also precipitated by the finding by Quintana et al. that the use of NOD/SCID mice might underestimate the frequency of tumourigenic cancer cells and that the use of the NOD.Cg.Rag mouse was found to increase the detection of tumourigenic melanoma.
cells by several orders of magnitude, indeed one single melanoma cell was found to be sufficient for tumour formation [146]. We have also made use of this strain for our studies using established cancer cell lines. Our success with this strain for tumour lines has been much better than our use of other strains (1/2 samples tested was capable of forming a tumour). However, it should be noted that the other major optimisation was also employed for the experiment that resulted in a successful engraftment of tumour, the cleared and humanised fat pad assay. This assay makes use of clearing most of the endogenous mammary gland of 3-4 week old female mice and replacing it with a combination of irradiated and unirradiated human fibroblast cells. The use irradiated fibroblasts is because irradiated cells are thought to cause the breast microenvironment to be characterised by remodelling of the extracellular matrix proteins of the adipose stroma, including increased collagen synthesis and activation of TGF-β that leads to a more conducive environment for tissue engraftment [159-161]. Unirradiated cells are used in combination to aid the engraftment of the irradiated fibroblasts, and additionally provide for a microenvironment within the breast that is conducive to further engraftment of human tissue. We found that one primary sample (Q468) when engrafted into a prepared mammary fat pad was capable of sustained growth over a period of 125 days. This sample grew only when injected as whole tumour pieces as a similarly prepared littermate that was injected with single cells did not produce tumour growth. Furthermore, this sample when processed was found to be capable of culture in tumoursphere-promoting conditions. This validates the potential use of mouse models as an “in vivo incubator” to generate additional cells for further ex vivo testing. The final piece of optimisation was the use of slow-release oestrogen tablets to potentially provide the systemic hormonal environment that oestrogen receptor positive and oestrogen dependent tumours need to survive. Interestingly, after the fact it was found that Q468 was in fact ER negative; however, use of the oestrogen pellet is necessary before immunohistochemistry can be performed in case the tumour does require exogenous hormone. Future experiments exploring the growth of primary tumour samples utilising this technique might find the most successful engraftment rate to be reached utilising the NOD.Cg.Rag mouse, with hormonal stimulation, with the humanised cleared fat pad injection route and using whole tumour pieces that have only been mechanically disturbed. As a result of our low success rate at both culturing tumourspheres from primary samples and inducing engraftment of primary tumours in xenograft assays, we decided to investigate the growth of tumourspheres from established breast cancer cell lines.

### 3.4 Conclusions
Here we have shown that tumourspheres can be cultured from both primary breast cancer tissue and breast cancer metastatic tissue. These tumourspheres exhibit some of the properties of cancer stem cells – self-renewal as tumourspheres and proliferation. In the case of primary tissue samples proliferation was not possible beyond a certain number of passages as cells did terminally differentiate, indicating perhaps that other culture methods might do a better job of sustaining growth as tumourspheres or that these cultures lacked long-term proliferating cells. Alternatively, the finite length of time for growth in tissue culture could reflect the lack of time for transformation to occur that is seen in established immortalized cell lines or that specific cell-cell interactions that exist in vivo that are necessary for long term culture are lost in vitro. In the case of the metastatic breast cancer sample proliferation continued for more passages but also resulted in terminal differentiation. This perhaps indicates that metastatic cells are more highly enriched in cells that have some of the properties of cancer stem cells, and this would be in line with the current paradigm of cancer stem cells being responsible for metastatic disease. We have also demonstrated the ability to culture primary tumour samples in a mouse model, with the hypothesis that growth in a humanised mouse mammary microenvironment might provide better cell-cell interactions that are lost in vitro. This model required a considerable amount of optimisation, but future work investigating tumourigenic cells of breast cancer origin will certainly benefit from making use of this “in vivo incubator” system to enrich for tumourigenic cells. Future experiments investigating primary and metastatic breast cancer samples should be conducted with first using the in vivo incubator approach to enrich for cells, then investigating the properties of the cells that fulfill the functional and phenotypic characteristics of breast cancer stem cells both ex vivo and back in vivo through serial transplantation. Establishment of breast cancer cell lines straight in vitro has long been recognised as a difficult task, and this has been noted to be especially true for the case of samples that are not derived from pleural effusions. We did not have access to pleural effusion samples (the use of pleural effusion samples is worth exploring in our system in the future) and we experienced a decline in samples post 2007, likely as a result of patients presenting with smaller tumours, and hence less tumour sample for research purposes. As a result of these facts, coupled with the fact that our xenograft model was only optimized in late 2009, we began to investigate established breast cancer cell lines for tumoursphere growth. Further identification of cell lines that are capable of growth as tumourspheres and the functional properties of their growth in tumoursphere-promoting conditions are discussed in the next chapter of this thesis.
Chapter Four: Modelling Stem Cell-like Cells in Breast Cancer Cell Lines

4.1 Introduction

Stem cells are difficult to phenotypically describe as no definitive well-accepted positive marker exists for their isolation or discrimination from non-stem cells. Therefore, stem cells have been functionally defined. Stem cells represent a relatively quiescent population of uncommitted cells that retain the ability to divide throughout the lifespan of an organism, and which display extensive proliferative potential over time. Stem cells divide through symmetric division to give rise to another stem cell (self-renewal), or through asymmetric division to give rise to more committed progenitor cells. Progenitor cells divide more frequently than stem cells and thus can generate a large number of differentiated progeny, but have a more limited proliferative potential over time. Functionally identifying stem cells in a population of cells involves identifying the self-renewal capacity, the ability for extensive proliferation, and the ability to give rise to differentiated progeny (reviewed in [108]).

A growing body of evidence (referenced elsewhere in this thesis) supports the hypothesis that cancer is initiated and sustained by a population of cells with some of the characteristics of stem cells. These cancer stem cells or tumour-initiating cells are thought to be resistant to traditional treatments and drive tumour recurrence because of their stem cell-like properties. Therefore it is likely that an in vitro model for the validation and enumeration of stem cell characteristics (self-renewal, stem cell frequency and stem cell expansion rate) in primary tumour samples or cancer cell lines is necessary to design effective and innovative treatments targeting this population of cells. Additionally, it would be most beneficial if the in vitro model was capable of predicting the growth characteristics of tumours in vivo – either in patients or in a xenograft model.

The Neurosphere assay (NSA), first identified in relation to adult neural stem cells [75], is the preferred method for isolation and expansion of somatic and cancer stem cells. This in vitro method, first identified by Reynolds and Weiss in 1992, demonstrated that a small population of cells (less than 0.1% of total cells) isolated from the adult murine striatum could proliferate and generate multicellular structures – neurospheres. This culture system represents a serum-free system in which receptive cells are capable of expansion when stimulated with the appropriate
growth factors, such as epidermal growth factor (EGF) [162-164] and fibroblast growth factor 2 (FGF2) [165, 166]. In this culture system, most of the differentiated cells (greater than 90%) die during dissociation and passage. The mitogen-responsive cells, the stem cells, divide and form new spheres. The whole process can be repeated multiple times (typically at least three times or more) to demonstrate self-renewal capacity and expansion potential. This method has been used extensively in the study of central nervous system somatic stem cells and cancer stem cells (reviewed in [167]). Importantly, this method has proved useful in breast somatic stem/progenitor cell and breast cancer stem cell research and has led to the naming of spheres from these breast stem cells/cancer stem cells as “mammospheres” and alternatively as tumourspheres in the specific case of cancer [33, 69, 77]. The advantage of this assay is the accurate identification of stem cell activity. One of the applications of this assay is in measuring sphere forming efficiency, the frequency of spheres formed from a set number of plated single cells. This number is often taken to be an approximation of the frequency of stem cells present in the seeding population (if it is to be believed that each sphere is initiated by one stem cell). However, recently the limitations of this assay have been realised, such as its overestimate of stem cell frequency [163, 168]. A mathematical interpretation of the neurosphere assay has been proposed (Loic Deleyrolle and Brent A. Reynolds, McKnight Brain Institute, University of Florida, 2010, in submission) that allows actual measurement of stem cell numbers and stem cell symmetric division frequency.

In this mathematical model proposed by Deleyrolle and Reynolds, the fold expansion of cells over several passages is calculated. Fold expansion is defined as the increase in total number of cells compared to the initial starting amount. The fold expansion is different for each cell line but is consistent between passages when sampled at the same time point each instance (i.e. cell line A has an 8-fold expansion and cell line B has a 5-fold expansion that remains constant as long as sampling always occurs on day 5 after initial seeding). It is important to remember that in this culture system most of the cells die upon initial seeding within 24-48 hours. As a result, the fold expansion rate is dependent upon the growth factor-responsive cells proliferating, i.e. the stem cells. The fact that most cells die upon initial seeding and that cell lines can be passaged in this way indefinitely (taken to mean longer than the experiment goes on for, usually greater than 5-8 passages), indicates that long-term proliferating (LTP) cells, cells with the hallmark characteristics of stem cells, are responsible for maintaining the spheres over time. The frequency of LTP cells in a population is reflected in the fold-expansion rate (the slope of the growth curve), and as LTP cells divide by symmetric division to give rise to new LTP cells, it follows that the slope of the growth curve is a reflection of the number of symmetric divisions occurring over that
particular culture period (Figure 4.1). In this model, spheres are composed of LTP, short-term proliferating (STP) cells and non-dividing (ND) cells. This model assumes that the life of LTP cells is at least as long as the experiment (typically 5-8 passages), and that the life of STP cells is finite (less than the length of the experiment). STP cells are identified in this model as precursor cells, they cannot divide to form a LTP cell but can give rise to spheres that are composed of only STP cells and ND. These spheres; however, do not have cells capable of forming further spheres over additional passages. Hence, the long-term propagation of the population is reliant on LTP cells.

Serial passage of tumourspheres can be used to measure the fold expansion of particular lines that can be cultured under tumoursphere-promoting conditions. Serial passage involves culturing single cells as spheres, dissociating them after a set number of days (typically when spheres reach 120 μm in diameter of the long axis, around 5-7 days), seeding new single cells sampled from the dissociated population, and allowing the growth factor-responsive cell population to give rise to new spheres. The process is repeated as many times as necessary, however, the number of passages should be higher than three to accurately define a population of cells to have LTP cell activity and to compare their activity between populations [163, 168]. Fold expansion, $F$, can be calculated by

$$F = T_f / T_i$$

in which $T_f$ is the cell count at the end of passage and $T_i$ is the cell count at the start of passage. If this number is stable over several passages, it indicates that the initial numbers and final numbers of LTP, STP, and ND cells are the same at each passage. Since each LTP cell generates one sphere and only LTP cells can create another LTP cell, it follows that the fold expansion is a reflection of the number of LTP cells in a LTP-derived sphere. Only LTP cells produce other LTP cells, thus, the rate of growth of LTP cell numbers is proportional to the total numbers of LTP cells ($F$). This is not influenced by the rate of asymmetric division, only symmetric division. Therefore, the symmetric division rate of LTP cells ($K_{II}$) is influenced by the fold expansion ($F$) for a particular time in culture ($t_f$) and can be represented in the following equation

$$K_{II} = \ln(F) / t_f$$
Figure 4.1. **Tumoursphere assay model.** (a) Long-term proliferating (LTP) cells give rise to spheres comprising LTP, short-term proliferating (STP) cells and non-dividing (ND) cells. Upon passage spheres are dissociated and only LTP cells survive to renew a new spheres at a one LTP to one sphere ratio. LTP cells can either not symmetrically divide (i.) or undergo symmetric division to produce new LTP cells (ii. = one symmetric division and iii. = three symmetric divisions). (b) The LTP symmetric division rate influences the slope of the curve generated from serial passage of tumourspheres (i. = one fold expansion; ii. = two fold expansion; and iii. = four fold expansion). [Modified figure from Deleyrolle and Reynolds, 2010, in submission]
Hence, taking the natural logarithm of the fold expansion and dividing by the time in culture will yield the symmetric division rate of LTP cells forming LTP spheres.

Establishing the capacity for growth of breast cancer cell lines in tumoursphere-promoting conditions is an important aspect of research regarding cancer stem cells. We hypothesise that culture in tumoursphere-promoting conditions and application of the mathematical model for determining symmetric division rate of LTP cells will accurately identify a population of cells with characteristics of breast cancer stem cells from established breast cancer cell lines. Here, we investigated a panel of breast cancer cell lines for their capacity to culture in tumoursphere-promoting conditions. We investigated the growth potential of several breast cancer cell lines in adherent and tumoursphere conditions and enumeration of the sphere forming efficiency for some of these lines. We also validated the mathematical model for use in determining the fold expansion and symmetric division rate of LTP cells, and demonstrated that the symmetric division rate for a cell line has predictive value for determining the tumorigenicity of some breast cancer cell lines. We further investigated other properties of cancer stem cells such as: (i) the relationship between the slow cycling population and tumorigenicity and sphere forming efficiency; (ii) comparisons between tumorigenicity of adherent cells and tumoursphere-derived cells; and (iii) serial passage in mice for some tumoursphere lines. Overall, these results support the use of tumoursphere-promoting conditions as a method for investigating breast cancer stem cells.
4.2 Results from established breast cancer cell lines

4.2.1 Screening of 25 breast cancer cell lines for capacity for growth in tumoursphere conditions. In order to gain a more comprehensive understanding of cell lines in vitro sphere-formation, we tested the ability of 23 breast cancer cell lines and 2 normal breast epithelial cell lines to form spheres. HBL-100 and SVCT are considered non-malignant, having been derived from non-tumourigenic tissue [169, 170]. Additionally, HBL-100 and MDA-MB-435 cell lines are no longer considered by many to be breast cancer cell lines, given evidence for male [171] and melanoma [172] origins respectively. RHBI cells, is a breast cancer cell line not previously described in the literature that is tumourigenic in mice. In these culture conditions, cell lines were classed as “non-sphere forming” (-) and “proficient-sphere formers” (+). Cell lines were tested for their ability to form spheres using both human and mouse proliferation supplement. In only one case was the use of human proliferation supplement deemed absolutely better than mouse proliferation supplement for the growth of spheres. BT-20 cells were incapable of culture as spheres with mouse but could be cultured as spheres with human proliferation supplement. Results are summarized in Table 4.1. Representative spheres are shown in Figure 4.2. Spheres are assessed as multi-cellular structures, and distinguished from clumps of cells by spheres 3D characteristic.
Table 4.1. Sphere Forming Capacity (SFC) of 25 established cancer cell lines. SFC was assessed in cells lines using both mouse and human proliferation supplement (StemCell Technologies Inc). Ability to culture as tumourspheres beyond passage 5 (P5) is also reported.

Adherent growth media conditions: foetal calf serum (FCS) (Hyclone, Logan, UT, USA); DMEM (Gibco, Invitrogen, USA); F12 (Gibco, Invitrogen); RPMI (Gibco, Invitrogen); I = bovine Insulin (Sigma); HC = hydrocortisone (Sigma); EGF = epidermal growth factor (Sigma).

Cell lines were kindly donated by MB: Melissa Brown, School of Molecular and Microbial Sciences, University of Queensland, AUS; LH: Lily Huschtscha, Westmead, The University of Sydney, AUS; GCT: Georgia Chenevix Trench, Queensland Institute of Medical Research, AUS; TB: Tony Blick, St. Vincent’s Hospital, Melbourne, AUS; GL: Gillian Lehrbach, Garvin Institute of Medical Research, Sydney, AUS; MM: Mike McGuckin, Mater Medical Research Institute; CS: Chanel Smart, UQ Centre for Clinical Research, AUS; or were sourced directly from the ATCC (Rockville, Maryland, USA).
Figure 4.2. Four times magnification sphere photomicrographs from a variety of cell lines. Representative day 5-7 spheres from luminal, basal, or mixed phenotype cancer cell lines from between passage 1 and passage 3. (a) KPL-1 (b) BT-474 (c) MCF7 (d) T47D (e) ZR-75-1 (f) Hs578T (g) B-T20 (h) HBL-100 (i) MDA-MB-436 (j) SUM-159-PT and (k) RHB1. Black bars represent 100 μm.
4.2.2 Growth of breast cell lines as spheres and adherent cultures. Five basal-like and five luminal-like breast cancer cell lines were grown in tumoursphere conditions with matched adherent cultures. Representative growth curves are shown in Figure 4.3a-b. All cell lines were capable of culture to at least 8 passages under adherent conditions. All cell lines except for Hs578T (grown to P2) and SK-BR-3 (grown to P4) were capable of culture as tumourspheres to at least 5+ passages (BT-20) and often times greater than 8 passages (all remaining cell lines).

4.2.3 Application of the mathematical model to a panel of breast cancer cell lines. Five basal-like and five luminal-like breast cancer cell lines were grown in tumoursphere conditions with matched adherent cultures. Each cell line displayed a particular fold expansion rate and $K_{II}$ (Figure 4.4a and 4.4b). A correlation test comparing the average of the fold expansion rate for spheres to matched adherent cells demonstrated a significant trend, $p = 0.002$ (Figure 4.4c). A correlation test comparing the average sphere $K_{II}$ to the average adherent $K_{II}$ demonstrated a significant trend, $p = 0.0016$ (Figure 4.4d).

4.2.4 Sphere forming efficiency does not increase over serial passage of spheres and is not correlated with fold expansion or $K_{II}$. Six cell lines were analysed for SFE over 5 serial passages (Figure 4.5a-b). Results are shown as combined data from; two independent experiments for MCF7, MDA-MB-436, and BT-474 (P1-P5); three independent experiments for SUM-159-PT (two for P1-P2 and one for P1-P5); and two independent experiments for HBL-100 and KPL-1 (one for P1-P4 and one for P1-P5). Data for each passage represents the combined SFE for a dilution of cells (5000, 1000, 500, and 100). SFE over serial passage was found to not significantly (in this case p value less than 0.03) change for SUM-159-PT, MCF7, or BT-474 spheres. SFE was found to significantly decrease for HBL-100, MDA-MB-436 and KPL-1 cells, $p \leq 0.0001$. Statistics used a one-way analysis of variance with a post-test for linear trend. Overall SFE ± SEM between P1-P5 was also calculated (HBL-100 = 5.1 ± 3.5; SUM-159-PT = 2.5 ± 2.0; MDA-MB-436 = 2.3 ± 1.9; MCF7 = 4.1 ± 2.5; KPL-1 = 3.8 ± 2.7; and BT-474 = 2.6 ± 2.3). Comparison of SFE at P1 (Average of 3-5 independent experiments) to average fold expansion revealed no correlation (Figure 4.6a). Comparison between the overall SFE and average fold expansion revealed no correlation (Figure 4.6b). Comparison of SFE at P1 (Average of 3-5 independent experiments) to average $K_{II}$ revealed no
correlation (Figure 4.6c). Comparison between the overall SFE and average $K_{II}$ revealed no correlation (Figure 4.6d).

Figure 4.3

**a**

Growth curves for adherent and tumoursphere cultures. Representative growth curves of spheres (circles) and adherent cells (squares). Growth curves are calculated from triplicate cultures. (a) Basal-like lines and (b) luminal like lines.
Figure 4.4. Mathematical model application to a variety of breast cancer cells and comparison to growth as adherent cultures. (a) Fold expansion of ten sphere cultures representing five basal-like (dark columns) and five luminal-like (light columns) breast cancer cell lines. (b) $K_{ll}$ for the same cell lines. Columns represent mean and error bars are SEM. (c) Average fold expansion for spheres plotted on the x-axis and for matched adherent cells plotted on the y-axis. Correlation was significant demonstrating that the greater the fold expansion for adherent cell lines the greater the fold expansion for spheres, $p = 0.002$, **. (d) Average $K_{ll}$ for spheres plotted on the x-axis and for matched adherent cells plotted on the y-axis. Correlation was significant demonstrating that the greater the $K_{ll}$ for adherent cell lines the greater the $K_{ll}$ for spheres, $p = 0.0016$, **.
Figure 4.5. Sphere forming efficiency (SFE) for six breast cancer cell lines. (a) Basal-like and (b) luminal-like SFE for six lines between passage (P) 1 and P5. SFE was found to not significantly (p value less than 0.03) change over serial passage for SUM-159-PT, MCF7 or BT-474 spheres. HBL-100, MDA-MB-436 and KPL-1 demonstrated a significant decrease in SFE over passage, p ≤ 0.0001. One-way analysis of variance with a post-test for linear trend. Columns represent mean and error bars are SEM.
Figure 4.6. Comparison of sphere forming efficiency and the tumoursphere mathematical model for enumerating long term proliferating cells and symmetric division rate of long term proliferating cells. (a) Comparison of SFE at P1 to average fold expansion reveals no correlation. (b) Comparison of overall SFE (P1-P5) to average fold expansion reveals no correlation. (c) Comparison of SFE at P1 to average $K_{II}$ reveals no correlation. (d) Comparison between the overall SFE between P1 and P5 and average $K_{II}$ reveals no correlation.
4.2.5 Tumoursphere model of breast cancer stem cells and correlation with tumorigenicity. The three breast cancer cell lines used in this study could be serially passaged in the tumoursphere assay greater than 5 passages. The three lines demonstrated significantly different fold expansion rates over more than 5 passages (KPL-1 = 2±0.7, MCF7 = 1.5±0.2, and BT-474 = 1.2±0.2) and hence different numbers of LTP cells per spheres (Figure 4.7a). Similarly, the symmetric division rate was found to be significantly different for each line (Figure 4.7b). Subsequently, matched samples of these cells were implanted into immunocompromised mice at 1x10^6 cells per mouse subcutaneous and tumour progression was monitored. Tumour progression was found to be significantly different between the cell lines (Figure 4.7c). Importantly, the disease progression (determined by time that the animals needed to be euthanised because the tumour volume had reached 520mm^3) was directly and inversely correlated with the symmetric division rate of the LTP cancer cells (Figure 4.7d). Greater K_H was associated with lower mean survival. Mean tumour volume over the course of the experiment was also different for the three cell lines (Figure 4.7e).

4.2.6 Tumorigenicity of breast cancer cell lines. In order to further investigate the capacity of the tumoursphere assay to predict tumorigenicity of breast cancer cell lines we utilised a xenograft assay to assess the tumour take rate of several cell lines that were capable of forming spheres and also compared this information to the literature on tumorigenicity of breast cancer cell lines (reviewed in [173]). As shown in Table 4.2, several cell lines that are capable of culture as tumourspheres have not been shown by us or other researchers to be tumourigenic (or at best are weakly tumourigenic) in mouse xenograft assay, including Hs578T, HBL-100 and SK-BR-3. Conversely, several cell lines that have been shown to be tumourigenic in the literature were not found to be capable of growth in the tumoursphere culture conditions outlined here. One cell line that we investigated, MDA-MB-436, was found to be tumourigenic but the literature has previously reported this line to be non-tumourigenic [174]. Importantly, we have shown here, for the first time to our knowledge, that RHB1 cells are tumourigenic. Collation of data on cell line in vivo tumorigenicity using various mouse xenograft studies including work presented in this thesis and the work summarised by Lacroix [173] failed to stratify sphere-forming from non-sphere forming cell lines. This indicates that the tumoursphere assay does not necessarily discriminate in its capacity to culture tumour initiating or non-tumour initiating cells – i.e. the spheres formed in the tumoursphere assay are not necessarily tumourigenic, tumorigenicity is a different characteristic of the individual cell lines.
4.2.7 Adherent and tumoursphere-derived tumours demonstrate similar growth and survival kinetics in a xenograft assay. In order to determine if there are any differences in the growth of tumours derived from adherent or tumoursphere cultures, we compared the two for several cell lines in xenograft assays. Results demonstrate that there is no difference between the two culture methods in terms of tumour growth or percentage survival following injection of tumours into NOD.Cg.Rag mice for the cell lines SUM-159-PT, ZR-75-1, and BT-474 (Figure 4.8).

4.2.8 Increased tumour growth upon serial transfer of SUM-159-PT cells for both adherent and tumoursphere-derived cells in a xenograft assay.

In order to investigate the effect of serial passage of cells in mice, we used one cell line, SUM-159-PT, to serially passage three times in NOD.Cg.Rag mice. Tumour growth was detected earlier for both adherent and tumoursphere-derived cells upon the second (P2) or third (P3) passage in mice compared to the first establishment of tumours (P1) (Figure 4.9a). Nonlinear fit modelling using Prism software indicated that P2 and P3 spheres or adherent cells grew faster in mice compared to P1 spheres or adherent cells (***, p ≤ 0.0003). Additionally, a nonlinear fit model (Prism software), that excludes measurements on days where no tumour has been identified and days where tumour growth has reached a plateau, demonstrated significant differences in the growth curves between these cell lines. This was accomplished to demonstrate that the growth curves are different between these cell lines in addition to the time that it takes for tumours to develop. Hence, the model was applied to tumour measurements after the first day that tumours were measurable. This model demonstrated that the slope of tumour growth was significantly different between P1 (slope = 40±3.5) and P3 (slope = 75±4.5) adherent cells (p = 0.0014) but not between P1 and P2 (slope = 53±6.7) or P2 and P3. The slope of P1 spheres (25±2.6) was found to be significantly different to P2 (61±7.2) and P3 (49±6.3), (p < 0.0001, and p = 0.0003 respectively). P2 and P3 slopes were not significantly different. Likewise, percentage not at maximum tumour volume of the mice was determined to be reduced for P2 and P3 compared to P1 (Figure 4.9b). Logrank test showed significant difference in percentage not at maximum tumour volume between P1 and P2 tumoursphere injections (***, p = 0.0001) and between P1 and P3 tumourspheres injections (***, p = 0.0002). Likewise, a difference was noted for adherent cultures between P1 and P2 (***, p = 0.0003) and P1 and P3 (***, p = 0.0009). No difference was noted between either adherent or tumoursphere for percentage survival between
P2 and P3. No difference was noted between adherent and tumoursphere for percentage not at maximum tumour volume at the same passage in mice. P2 tumoursphere-derived tumours were also used determine the dose dependency of tumour establishment, with $1 \times 10^6$, $1 \times 10^5$, and $1 \times 10^4$ cells injected into mice, $n = 4$. Growth curves (Figure 4.9c) and percentage of mice not at maximum tumour volume (Figure 4.9d) demonstrated three different results for the three groups. Logrank test shows that $1 \times 10^6$ and $1 \times 10^4$ cells injected are significantly different (*, $p = 0.01$) for percentage not at maximum tumour volume. No differences between the remaining groups were found, $n = 4$ mice.

4.2.9 Slow-cycling ZR-75-1 tumoursphere-derived cells are as tumourigenic as fast-cycling cells. In order to investigate the difference between slow and fast cycling cells in tumourspheres, we made use of sorting CFDA SE labeled cells for assessing different populations (slow, medium, and fast cycling) (Figure 4.10a). At day 5, SFE and fold expansion at P1 in tissue culture were not found to be significantly different between slow, medium, and fast cycling cells. Slow SFE = $16 \pm 2.8$; Medium SFE = $16.1 \pm 2.4$; Fast SFE = $16.6 \pm 1.9$; Slow fold expansion = $5 \pm 0.8$; Medium fold expansion = $6.5 \pm 2.6$; Fast fold expansion = $5.2 \pm 1.3$. Tumour growth was not found to be different between slow and fast cycling cells at P1 in xenograft assays (Figure 4.10b) and P2 in a mouse (Figure 4.10c).
Figure 4.7. Application of the mathematical model to breast cancer tumourigenicity. (a-b) Three breast cancer cell lines were cultured in the Tumoursphere Assay. In these conditions the 3 cell lines displayed different expansion rate and LTP cell symmetric division frequency. ***, p ≤ 0.0001, t-test, 2 tails, n = 36. (c) Tumour growth was monitored over time between the 3 groups from which survival analysis was performed and graphed as percentage of animals that have not reach the maximal tumor size (520mm³) as a function of time. Logrank test, p < 0.0001 comparing the three populations to each other, n = 10. (d) Tumour progression after breast cancer cell s.c. transplantation was directly and inversely correlated to the rate LTP cancer cell expand via symmetric division. The dashed lines correspond to the 95% confidence band of the fit curve obtained by nonlinear regression. Coefficient R² = 0.996, p ≤ 0.05. (e) Mean tumour volume for the three lines plotted over the course of the experiment. Error bars are SEM.
### Table 4.2. Tumourigenicity of some established cancer cell lines.

Tumourigenicity was investigated for some breast cancer cell lines that are capable of sphere formation (Morrison) and compared to literature reporting tumourigenicity (Lacroix, 2004, citation as in text).

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>SFC</th>
<th>Tumourigenicity</th>
<th>Experiment</th>
</tr>
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<tbody>
<tr>
<td>HCC1937</td>
<td>-</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>-</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>BT-549</td>
<td>-</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
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<td></td>
<td></td>
</tr>
<tr>
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<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>MDA-MB-453</td>
<td>-</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>UACC-812</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RHB1</td>
<td>+</td>
<td>Yes</td>
<td>NOD.Cg.Rag−/− mice (2×10⁶) s.c. (3/3)</td>
</tr>
<tr>
<td>BT-20</td>
<td>+</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Hs578T</td>
<td>+</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>HBL-100</td>
<td>+</td>
<td>Weak</td>
<td>NOD.Cg.Rag−/− mice (2×10⁶) s.c. (0/3)</td>
</tr>
<tr>
<td>MDA-MB-435</td>
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<td>Yes</td>
<td>SCID/Beige mice (1×10⁶) s.c. (3/3)</td>
</tr>
<tr>
<td>MDA-MB-436</td>
<td>+</td>
<td>No</td>
<td>NOD.Cg.Rag−/− mice (2×10⁶) s.c. (3/3)</td>
</tr>
<tr>
<td>SUM-159-PT</td>
<td>+</td>
<td>Yes</td>
<td>NOD.Cg.Rag−/− mice (2×10⁶) s.c. (3/3)</td>
</tr>
<tr>
<td>KPL-1</td>
<td>+</td>
<td>Yes</td>
<td>NOD.Cg.Rag−/− mice (2×10⁶) s.c. (3/3) E2 dependent</td>
</tr>
<tr>
<td>BT-474</td>
<td>+</td>
<td>Yes</td>
<td>NOD.Cg.Rag−/− mice (2×10⁶) s.c. (3/3) E2 dependent</td>
</tr>
<tr>
<td>MCF7</td>
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<tr>
<td>SK-BR-3</td>
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<tr>
<td>T47D</td>
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<tr>
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<td>NOD.Cg.Rag−/− mice (2×10⁶) s.c. (3/3) E2 dependent</td>
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Figure 4.8. Comparisons for tumour growth and survival between adherent cells and tumourspheres for three cell lines. (a-b) First passage in a mouse (P1) SUM-159-PT tumour growth curve and survival analysis. Survival analysis was performed and graphed as percentage of animals that have not reach the maximal tumor size (520mm³) as a function of time. Adherent cells (ADH) n = 10, tumourspheres (NSA) n=10. (c-d) ZR-75-1 tumour growth curve and survival analysis. ADH n = 5, NSA n=10. (e-f) BT474 tumour growth and survival analysis. ADH n = 5, NSA n=10. Nonlinear fit modeling indicates no difference in tumour growth for spheres or adherent cells for SUM159 or BT-474. ZR-75-1 cells grown as adherent were found to grow quicker than spheres at passage 1 (p = 0.0017) Logrank test comparing the survival of mice after ADH or NSA injections demonstrated no significant difference. Experiment was done twice for SUM-159-PT (one representative result shown) and once for ZR-75-1 and BT-474.
SUM-159-PT cells demonstrate increased tumour growth after serial passage in mice for both adherent and tumoursphere-derived cells and there is a dose dependency for tumour growth. (a) Tumour growth for adherent cells (ADH) and tumoursphere-derived cells (NSA) upon first passage in a mouse (P1), or second and third passage (P2, P3). Nonlinear fit modeling indicates that P2 and P3 spheres or adherent cells grow faster in mice compared to P1 spheres or adherent cells (***, p ≤ 0.0003). No difference was found between spheres or adherent cells growth at P1, P2, or P3. (b) Survival analysis was performed and graphed as percentage of animals that have not reach the maximal tumor size (520 mm$^3$) as a function of time. Logrank test showed significant difference in % survival between P1 and P2 tumoursphere injections (***, p = 0.0002) and between P1 and P3 tumourspheres injections (***, p = 0.0002). Likewise, a difference was noted for adherent cultures between P1 and P2 (***, p = 0.0003) and P1 and P3 (***, p = 0.0009). No difference was noted between either adherent or tumoursphere % survival at the same passage in mice. (c) The P3 tumour growth curve for SUM-159-PT cells using three amounts of cells. Nonlinear fit modeling indicates that injections of 1e6 grow significantly faster than 1e5 or 1e4 (***, p ≤ 0.0001). No difference in growth was found between 1e5 and 1e4. (d) The % not at maximum tumour volume for the same populations of cells. Logrank test shows that 1e6 and 1e4 is significantly different (*, p = 0.01) for % survival. No difference between the remaining groups, n = 4.
Figure 4.10. Slow cycling ZR-75-1 spheres are as tumourigenic as fast cycling cells. (a) CFDA-SE sort for the 15% slow cycling, 15% medium cycling, and 15% fast cycling cells. (b) Mean tumour volume for slow and fast cycling cells injected at 500K cells into NOD.Cg.Rag<sup>-/-</sup> mice, n = 4. All mice were euthanised on day 21. Nonlinear fit modeling of the data indicates that slow and fast cycling cells initiated and sustained tumour growth at the same rate. (c) Mean tumour volume upon second passage in a mouse at 100K cells, n = 1. No statistics performed for this as animal numbers were too low.
4.3 Discussion

In order to investigate the functional stem cell properties of breast cancer cells, we made use of the tumoursphere assay. Tumourspheres are 3-dimensional multicellular non-adherent structures comprised of a small number of self-renewing cells, capable of further rounds of sphere formation upon serial passage, as well as a larger number of more differentiated progenitor cells and fully differentiated cells. Thirteen out of twenty-five (52%) immortalised established cell lines tested demonstrated the capacity to culture as tumourspheres. These lines represent a variety of breast cancer subtypes. The subsequent chapters outline further investigation into the subtypes of breast cancer cell lines that can form spheres, as well as functional, morphological and antigenic similarities and differences between tumourspheres of different subtypes of breast cancer. Here we have functionally characterised sphere growth from breast cancer cell lines by looking at sphere forming efficiency, fold-expansion and long term proliferating cell symmetric division rate, and tumorigenicity of cells in xenograft assays. These criteria are all hallmarks of breast cancer stem cells. The results demonstrate the potential use of the tumoursphere assay for culture and investigation of cells that have a stem cell-like phenotype. This assay therefore has an important place in the field of breast cancer stem cell research.

The culture of breast cancer cell lines as tumourspheres has been well studied (references of others works and additional description of our work elsewhere in this thesis). We tested a variety of breast cancer cell lines for their ability to culture as tumourspheres and noted that not all lines could be cultured as tumourspheres, and that even amongst lines that could be cultured as tumourspheres, there was much variation in the ability for sustained serial passage of tumourspheres. Sphere formation was observed in 13 out of 25 cell lines examined and included previously reported sphere-forming cells SUM-159-PT [83, 88], BT-474 [78], MCF7 [69, 77, 78, 82, 83, 90], SK-BR-3 [82], T47D [78, 175] and ZR-75-1 [78]. For the first time to our knowledge, we report sphere formation in KPL-1, MDA-MB-435, MDA-MB-436, HBL-100, Hs578T, and BT-20, but did not observe sphere formation in MDA-MB-231 [82, 90] and MDA-MB-453 [88] which has been previously reported. We also report here the capacity for RHB1 cells to form spheres. RHB1 cells are a tumourigenic breast cancer cell line that has not been previously cited in the reviewed literature. Some of the cell lines tested demonstrated a high capacity for long-term culture as spheres (HBL-100, MCF7, KPL-1 and T47D for instance). Others, such as SK-BR-3 and Hs578T, can certainly be cultured as spheres but lack the ability for
sustained serial passage in tissue culture. In fact, the symmetric division rate of LTP cells for SK-BR-3 and Hs578T was found to be negative, perhaps indicating the complete lack of LTP cells in these cultures. The poor growth in tumoursphere-promoting conditions of certain cell lines could perhaps be due to such reasons as (i) lack of seeding numbers of LTP cells; or (ii) tumoursphere conditions not being exactly tailored to that individual cell line. Certainly, there could be other proliferation requirements that drive sphere formation different than those that we provided (proliferation supplement and EGF and FGF). For instance a variety of researchers make use of B27 supplement instead of proliferation supplement [33, 69, 78, 130] and this might result in some cell lines having a better or worse capability for growth as tumourspheres. Indeed, we found that the BT-20 cell line was incapable of growth as spheres when using murine proliferation supplement, but did generate classical-looking spheres when grown using human proliferation supplement. The difference between these two supplements is that they have either human or murine specific versions of mitogens. Additionally, it is possible that sphere formation in these poor serial passage cell lines is driven more by short term proliferating cells that lack the ability for sustained growth over time, and which ultimately lead to terminally differentiated cells incapable of growth as spheres. Interestingly, cell lines that had the poorest growth as adherent cells also demonstrated the poorest growth as spheres (Hs578T, SK-BR-3 and BT-474). The reverse was also true as T47D and HBL-100 demonstrated excellent proliferation as both adherent culture and sphere culture. Indeed, a correlation was found between the growth of adherent and sphere culture as assessed by both fold expansion and K_{H1}. This might indicate the overall growth of cells in both culture systems is sustained by the same population of cells, in that growth under tumoursphere-promoting conditions mimics the growth in adherent conditions but to a lesser degree. This is the first time, to our knowledge, that the correlation between growth as adherent cells and growth in tumoursphere culture has been reported for breast cancer cell lines.

One of the most fundamental functional characteristics of a stem cell is the ability of that cell for self-maintenance or self-renewal [5]. Previous research has supported the view that the number of mammospheres (or tumourspheres) formed upon serial passage in mammosphere/tumoursphere-promoting conditions reflects the self-renewal of mammary stem cells (or cancer stem cells), while the size of spheres formed is a reflection of progenitor cell proliferation [33]. Sphere forming efficiency has subsequently been used by a variety of researchers to demonstrate the effects that knocking down expression of genes such as BRCA1 [44], overexpressing genes such as HER2/neu [85], or selecting for molecular signature such as ALDH1 activity [88] has on self-renewal of putative stem/progenitor cells. These experiments demonstrated that the self-renewal
capacity of cells forming spheres could be assessed in a SFE assay and biological factors affecting this capacity can be experimentally tested. Of course, these experiments also utilised other methods to investigate cancer stem cells such as investigating the effect of their treatment/selection on tumorigenicity in mice, or comparing to phenotypic interpretations of breast cancer stem cells. Hence, self-renewal is an important aspect of research into breast cancer stem/progenitor cells and can, in some situations, be a useful assay to investigate putative markers of stem cells. In order to enumerate the self-renewal capacity of cell lines, we tested 6 cell lines for SFE over 5 passages. For three of these lines (SUM-159-PT, MCF7, BT-474), SFE was found to be stable and for the other three (HBL-100, MDA-MB-436, KPL-1), SFE decreased over passage. The result in the case of MCF7 is in contradiction to the previously reported increase in SFE over passage [77]. It is important to investigate not only primary passage tumoursphere formation but subsequent passage SFE as well. This is important to show: (i) that long term culture with sphere formation is possible; (ii) whether or not subsequent passage further enriches for sphere formation; and (iii) in the case of investigating treatments (differentiation or elimination of the cancer stem cell population), the treatment is affecting LTP cells and not just STP and ND cells. In the case of the lines in which SFE was found to decrease over passage, it should perhaps be noted that this decrease, although significant, did not represent a substantial decrease and could be the result of experimental error in determining SFE. If that is the case, it could be taken that SFE stays relatively constant over passage. This indicates that the tumoursphere assay enriches/selects/identifies for sphere forming cells at passage one, but this number of cells (stem cells or LTP) stays relatively the same through subsequent passages. This result is in accord with the mathematical model in which LTP cell numbers are thought to remain constant over time.

The fact that SFE stays constant over serial passage could indicate the presence of LTP cell activity in these cell lines since they are capable of self-renewal at a relatively stable rate. Sphere formation has often been used to assess self-renewal efficiency of cancer stem cells, and hence has been for some researchers seen as a surrogate for identifying stem cell numbers/frequency. However, SFE is an overestimate of cancer stem cells or LTP cell numbers [163, 168] and does not overestimate LTP cell numbers in a uniform (or useful) manner. We found no correlation between SFE and either fold-expansion rate or $K_{II}$. The fact that SFE overestimates LTP cell presence can be demonstrated in the finding that SFE at passage one for the three luminal-like lines was found to be between 3-4.4%, higher than the fold expansion rates found for these cell lines between 1.2 and 2. The same was found for the basal-like lines of a SFE between 3.4-8.4%
and a fold expansion rate being less at 1.7-2.5. These overestimates were also found when looking at the overall SFE between P1 to P5. However, HBL-100 had the highest SFE and the highest fold expansion rate (and Kd) indicating that the two methods can to a certain degree demonstrate the same result. This overestimation of the SFE assay of LTP cells could be due to reasons such as counting spheres that were initiated by STP and not LTP cells as there would be no way to visually tell which is which, without making use of a colony-forming assay system. The colony-forming assay system is based on the assumption that STP cells exhibit limited proliferative capacity compared to LTP cells, suggesting that large colonies (formed by LTP) have a greater proliferative potential and exhibit all of the key tissue culture stem cell characteristics (extensive self-renewal, generation large number progeny and multi-lineage differentiation potential) compared to smaller colonies (formed by STP), which do not exhibit these stem cell criteria [168]. Overall, these findings suggest that SFE can be a useful assay to show self-renewal of a population of cells comprising spheres; however, determination of fold-expansion rates over multiple passages might be a better way to enumerate stem cell numbers.

Three luminal-like cell lines were used to test the application of the mathematical model for predicting tumorigenicity in mouse models. The three breast cancer cell lines reported here (MCF7, BT-474, and KPL-1) were all of a luminal subtype; capable of forming spheres; capable of being passaged greater than 5 passages; and had similar sphere forming efficiency at passage one of between 3-4.4%. However, application of the mathematical model revealed differences between the three cell lines in terms of numbers of LTP cells, symmetric division rate of LTP cells, and influence of these factors on tumour progression in a xenograft model. It was found that the cell line with the highest fold expansion and symmetric division rate of LTP cells (KPL-1) also had the quickest tumour progression in vivo. The opposite was true for the cell line (BT-474) with the lowest fold expansion and LTP cell symmetric division rate. These results suggest that: (i) the LTP cell symmetric division rate measured in vitro for breast cancer cell lines using the tumoursphere assay can be used to predict tumor progression in vivo; (ii) the tumoursphere model can be applied in breast cancer research; and (iii) malignant LTP cells are responsible for driving tumour expansion. This model will need to be further validated in additional breast cancer cell lines. Application of this model to another tumour type (glioblastoma) that is capable of forming spheres has demonstrated similar results (Deleyrolle and Reynolds, 2010, currently in submission). Additionally, it would be useful, but beyond the scope of this thesis, to assess the relationship between LTP symmetric division in vitro and the tumour-initiating cell frequency in vivo through a limiting dilution assay. This experiment could be planned to incorporate the three
cell lines already investigated in the reported data here (MCF7, BT-474, and KPL-1) and compare the frequency of cells that are required to initiate a tumour. These assays are done over three or more dilutions of cells injected into animals and usually include a higher dilution where all animals develop a tumour, and lower dilutions where some animals develop a tumour and others do not. It would be quite interesting if the frequency of tumour-initiating cells in this assay was higher for (in order) KPL-1, MCF7, and BT-474 as this would lend credence to the results we found showing that tumour growth was correlated with LTP symmetric division. On a related note, preliminary results comparing tumour initiation for MCF7 tumourspheres to adherent cells in a dilution assay (500K, 100K, and 50K cells) has shown that tumourspheres have a higher frequency of tumour initiating cells than adherent culture. This suggests that the sphere assay does enrich for cancer stem cells compared to adherent culture. It would be desirable to do a more comprehensive analysis of this result in the future.

This mathematical model will find broad applications within the fields of somatic stem cell research and cancer stem cell research. One application will be for investigating ways that stem cells regulate self-renewal – the signaling pathways involved and the endogenous and exogenous signals that are required. Functionally identifying stem cells in this way could lead to better ways to phenotypically isolate and discriminate stem cells from other cells. This could find application in somatic stem cell studies of both murine and human organ systems, including the breast and the brain. Understanding stem cell self-renewal will be a critical step towards the development of rational treatment of diseases related to aging or trauma of the central nervous system. Additionally, this methodology will find application as a tool for investigating cells with cancer stem cell characteristics. The cancer stem model identifies cells with stem-cell like characteristics as being important for cancer initiation and progression. It is the stem cell-like characteristics of these cells that allow them to resist treatments such as chemotherapy and radiation therapy. Therefore, use of this model will allow investigators to identify cancer stem cells and have an in vitro model to test therapeutics or to test candidate stem cell markers (Deleyrolle and Reynolds, 2010, currently in submission). Certainly, this model could be applied to enumerating the LTP cells present in populations of cells selected for candidate stem cell markers such as ALDH1, CD49f, HER2, MUC1, CD44+CD24-. It is possible that this model could even be applied to patient tumour samples to investigate therapies that are likely to either differentiate cancer stem cells or eliminate them and then to use this knowledge to develop tailored therapies.
The finding that growth in tumoursphere-promoting conditions could be used to predict the tumour growth in mouse models prompted us to further investigate whether there was a correlation between the tumorigenicity of a breast cancer cell line and its capacity to culture in sphere-promoting conditions. This investigation revealed that there is not a direct connection between the ability to culture as tumourspheres and tumorigenicity. This indicates that the tumoursphere assay does not necessarily discriminate in its capacity to culture tumour initiating or non-tumour initiating cells – i.e. the spheres formed in the tumoursphere assay are not necessarily tumourigenic, rather tumorigenicity is a different characteristic of the individual cell lines in the particular mouse model under investigation. For instance, HBL-100 has a high SFE in comparison to the other cell lines tested and can be serially cultured as spheres for over 8 passages. However, this line has been found by us and others to be non-tumourigenic. Other cell lines that are highly tumourigenic such as MDA-MB-231 were found to be incapable of culture as spheres in our method. Culture in the tumoursphere assay allows enumeration of the symmetric stem cell division rate and can be used to demonstrate the presence of stem cells driving proliferation in vitro. The cells that are driving this propagation in vitro can either be tumourigenic in mice or not. For the cells that are tumourigenic (as described by the MCF7, KPL-1, and BT-474 experiment previously outlined), the application of the tumoursphere assay mathematical model does have some predictive value in determining tumorigenicity.

We further investigated tumorigenicity by comparing growth of adherent derived cells and tumoursphere-derived cells. The growth curve of adherent cells in tissue culture is steeper than the growth curve of cells grown as spheres. We found a correlation between growth as adherent cells and growth as tumoursphere cells. This might suggest that the overall growth of cells in both culture systems might be sustained by the same population of cells. This prompted us to investigate the tumour growth of both of these populations in vivo. We hypothesised that tumoursphere-derived cells would be more tumourigenic than adherent cells. It was found that for the three cell lines tested (SUM-159-PT, ZR-75-1, and BT-474), tumour growth and survival of mice post tumour cell injection was similar between adherent cells and tumoursphere-derived cells. This suggests that: (i) although tumoursphere growth in vitro appears to be less proliferative than adherent culture conditions, growth in mouse tumour models is the same; and (ii) that the same LTP cells responsible for growth of spheres in vitro are also the malignant cells causing tumour growth in vivo. These results further validate the use of the tumoursphere assay for investigating breast cancer stem cells. Additional cell lines will need to be tested comparing adherent cells and tumoursphere-derived cells growth in vivo. We also tested the ability of one
cell line (SUM-159-PT) for serial transfer in mice. This cell line was chosen because of the quick growth of tumours from both adherent and tumoursphere conditions. SUM-159-PT cells (either adherent or tumoursphere-derived) dissociated from tumours upon second and third passage in a mouse demonstrated an increase in the tumour growth curve and a decrease in survival compared to cells implanted the first time in a mouse. This indicates that growth as tumours in mice enriches or selects for cells that are more tumourigenic upon second or third passage in mice. However, there was no difference noted between the second and third passage tumour growth in mice or between adherent or tumoursphere-derived cells. We had hypothesised that tumoursphere-derived cells may be more tumourigenic in the long term (over serial passage) compared to adherent cells. We were only able to investigate one cell line for this experiment in numbers of mice that were statistically significant. Additional cell lines need to be tested in serial transfer studies. Serial passage with resulting tumour growth in secondary mice was conducted for both BT-474 (n = 3 mice) and MCF7 (n = 5 mice) for spheres and adherent cells, and indicated that other cell lines grown as spheres are capable of serial transfer in mice at a similar rate as adherent cells (data not shown). Future experiments will be needed to further explore the sustainability of serial transfer of adherent and tumoursphere-derived cultures, and determine if long term serial transfer capacity resides in cells grown as tumourspheres, adherent cells, both populations, or neither population. P3 tumour growth was also investigated for tumoursphere-derived cells at different amounts of cells 1x10^6, 1x10^5, and 1x10^4. It was determined that injections of cells at 1x10^6 resulted in a significantly faster tumour growth curve than the other two dilutions, which were not significantly different to each other, and validated our previous and subsequent usage of 1x10^6 as our standard amount of cells for injection. However it should be noted that only four mice were used for this analysis in one experiment and hence this finding is not conclusive.

The stem cell model predicts that cancer is driven by mutated adult stem or progenitor cells. Since adult stem cells are slow dividing, long lived cells, it has been thought that they would be susceptible to accumulation of multiple mutations over time, leading to the acquisition of traits of cancer stem cells such as self-renewal, differentiation and carcinogenesis [176-178]. As a result of this, it has been thought that cancer stem cells might also display a slow dividing phenotype or be in an unusual phase of the cell division cycle. It has been postulated that this might be the case for cancer stem cells in both primary tumours in vivo and for cells from cancer cell lines. Further investigation of the cell cycle phenotype of tumoursphere-derived cells and adherent cells is explored in later chapters of this thesis. Here, we investigated the cellular division for ZR-75-1
cells using CFDA SE to sort out slow, medium and fast cyclers based on the top, middle, and bottom 15% of cells expressing CFDA SE in order to assess these different populations for sphere formation ability and tumour formation. Our results indicate that SFE and fold expansion in tissue culture was not significantly different for these three populations. Previous results using a different cell line (MDA-MB-435) also demonstrated that these three populations did not have a significantly different SFE (data not shown). Furthermore, comparison of tumour volume in a xenograft assay demonstrated no difference for slow or fast cycling ZR-75-1 cells upon first passage in mice. Subsequent passage in mice was only conducted for one mouse each but showed that slow cycling cells are at least as capable as fast cycling cells in forming tumours. Importantly, these results indicate that slow cycling cells and fast cycling cells both are composed of cells that are capable of self-renewal and initiation of tumours. This work was exploratory and should be validated with more replicates. Future experiments will need to validate these results for additional cell lines.

4.4 Conclusions

Here we have shown that tumourspheres can be cultured from some breast cancer cell lines but not others. These tumourspheres exhibit some of the properties of cancer stem cells – self-renewal and sustained proliferation. Importantly, we have shown that growth of tumourspheres from established cell lines correlates with growth of cells as adherent cultures. SFE was determined to not increase over serial passage and was found to be an overestimate of the numbers of LTP cells present in tumoursphere culture. Application of a mathematical model for enumerating stem cell/LTP cell numbers and symmetric division rate of LTP cells in tumoursphere culture was used and shown to be predictive of tumorigenicity in mice. We further validated some other properties of cancer stem cells such as the ability for slow cycling cells to form spheres, serial transfer of tumoursphere-derived cells in mice, and comparison of growth of adherent and tumoursphere-derived cells in xenograft assays. Overall, the growth of adherent and tumoursphere-derived cells as tumours in mice was found to be similar, indicating that although growth in vitro is different, in vivo tumorigenicity is quite similar between the two culture conditions. Further research is warranted to investigate the effect of serial passage of tumoursphere-derived cells compared to adherent cells in mouse models. Future studies will need to validate the application of this model to a host of other cancer types in addition to further validation for breast cancer. In particular, the optimisation of the isolation and culture of human breast cancer primary cells in the tumoursphere assay would be highly advantageous. To date, the
culture of primary human breast cancer cells in the tumoursphere assay has been problematic. Indeed, the culture of breast cancer cell lines from primary patients has long been recognised as a difficult task. However, if more conducive growth conditions were identified for the long-term culture of human primary tissue in the tumoursphere assay, this model would be an ideal in vitro test to identify treatments capable of eliminating LTP cells (perhaps by immunotherapy or biotherapy) or differentiating them so they are no longer capable of sustaining tumour growth. This application could have a tremendous impact on clinical practice and patient welfare. Finally, within our system, we have identified that sphere forming cells are a rare cell within some cancer cell lines, but we have not phenotypically isolated them from the bulk of the sphere. Investigating the phenotypic differences between sphere forming cells and the bulk of the cells forming a sphere will be an important future avenue of research. Further identification of the functional and phenotypic properties of tumourspheres grown from established cell lines is discussed in the next chapter of this thesis.
Chapter Five: Characterising Basal-like and Luminal-like Tumourspheres and Investigating Phenotypic Characteristics of Tumourspheres

5.1 Introduction

Breast cancer cell biology research relies heavily on *in vitro* and *in vivo* models. Recent studies supporting the existence of a subpopulation of tumour-initiating or cancer stem cells have utilised such models [68, 69, 77, 85, 88, 119]. *In vitro* clonogenicity, differentiation and sphere formation and *in vivo* xenograft tumorigenicity assays, often coupled to other techniques (*e.g.* FACS), can be used to demonstrate self-renewal, generation of phenotypically diverse tumour cell populations, increased tumour-initiating potential and resistance to traditional therapeutics – key attributes of cancer stem cells (reviewed in [108, 179]).

The molecular heterogeneity of breast cancer is being resolved with molecular subtyping, and now greater discretion is required in sample selection to permit the observation of subtype specific biology. Such characterisations give greater prominence to the “luminal” and “basal”-like taxonomy now applied to several breast cancer subtypes on the basis of similarities to normal luminal and basal cells respectively of the breast epithelium [72, 73]. This prompts closer investigation of the biology of each subtype with a view to using this knowledge in the development of more tailored therapies (reviewed in [180]).

Characterisation of large panels of established breast cancer cell lines, propagated *in vitro* from primary and metastatic breast cancer, has demonstrated similar heterogeneity to primary breast tumours mirroring most of their important genomic abnormalities and variety of transcriptional profiles [150, 181, 182]. Cell lines may be criticized as models of breast cancer for their “unnatural” ability or selection to grow *in vitro*, their age and undetermined time and condition in culture and their increased rate of genetic alteration. Their many advantages, however, include the ability to reproducibly and economically manipulate them *in vitro* in multiple types of assays that are often not feasible or reliable using primary tumour cells or *in vivo* models (reviewed in [152]). For these reasons, *in vitro* assays using breast cancer cell lines still remain an important resource for research.
In vitro enrichment for normal breast stem cells in non-adherent, serum-free conditions was first introduced by Dontu et al. [33], as a variation on the method pioneered for cultivation of neural stem cells [183]. In these conditions, most cells undergo anoikis whilst a very small fraction of growth factor-responsive single cells divide and generate spheroid structures termed mammospheres/tumourspheres. Sphere formation was soon reported in cells derived from primary breast tumour [69, 79], metastatic sites [82] and breast cancer cell lines [69, 77, 82-84] and these tumourspheres were shown to enrich for tumour-initiating cells by in vivo xenograft assays. As a result, the tumoursphere assay gained popularity as a measure and model for studying breast cancer stem cells in vitro. The model was used to identify potential breast cancer stem cell regulators and test the effects of their in vitro manipulation on sphere-forming efficiency or tumorigenicity using overexpression, knockdown or antagonist experiments. To date, such studies have identified HER2 [85, 86], CD49f [77], PTEN [87], EpCAM [68, 83] and ALDH1 [84, 88] as important cancer stem cell markers. Recently, MUC1 expression has been reported on the side population cells from MCF7 mammosphere cultures demonstrating for the first time MUC1 expression on cells that have some of the functional characteristics of cancer stem/progenitor cells [130]. Additionally, the phenotype of CD44+CD24- cells has been identified for tumorigenicity of human metastatic samples in an in vivo model [68], ability to form mammospheres and undergo self-renewal in vitro [69], resistance to radiation [90] slow-cycling nature and resistance to chemotherapy [83], and enhanced metastatic and invasive characteristics [91, 92]. However, the heterogeneity of CD44/CD24 phenotypes in breast cancer stem cells has recently been explored by other investigators questioning the reliance on CD44+CD24- cells as the definitive phenotype of stem cell-like cells [83, 93, 94]. Fillmore et al. found a correlation between tumorigenicity and sphere formation in the breast cancer cell lines tested but this was related to the CD44+CD24-EpCAM+ population of cells indicating that tumorigenicity might not necessarily be restricted to the CD44+CD24- population [83]. Honeth et al. examined 240 human breast tumours and demonstrated that the CD44+CD24- phenotype is associated with basal-like tumours - characterised as negative for HER2, ER, and PR, and particularly that this phenotype was associated with BRCA1 hereditary breast cancer [94]. They showed that there is considerable heterogeneity both within and among tumours in the expression of CD44/CD24 indicating that tumorigenicity might not necessarily be restricted to CD44+CD24- cells. They also did not find any association between the CD44+CD24- phenotype and distant disease-free survival or factors such as tumour size, nodal status, or S-phase fraction that have been linked with clinical outcome. The case for CD44+CD24- as a putative cancer stem cell marker exemplifies the use of multiple difference sources of samples and models to investigate the breast cancer stem cell hypothesis.
Clearly more phenotypic characterisation will be required to further define the markers expressed by BCSCs. This is an important endeavour in order to further isolate BCSCs for research and for targeting using treatments.

The biological significance of breast cancer cell line sphere-forming data must be interpreted with care, noting that breast cancer cell lines are an in vitro resource modelling breast cancer just as tumoursphere formation is a functional assay used to demonstrate critical stem cell-like attributes of populations of cells and which appears analogous to tumour-initiation. To date, most studies using breast-cancer cell lines in sphere formation have used small panels of cell lines often too small to reliably interpret data in the context of the heterogeneity of cell line phenotypes [69, 77, 82]. Little too has been reported on the morphological differences of spheres formed from different sources, possibly misleading the field that these structures are homogenous. Molecular characterisation of breast cancer tumourspheres is long overdue for our understanding of the heterogeneity and differentiation within the structures themselves and thereby the functional significance of this assay. In this study, we screen a large panel of breast cancer cell lines for sphere-forming ability and compare these data to published functional, phenotypic and genotypic information for these cells lines. To assess the functional, morphological and phenotypic properties of tumourspheres we analysed three established basal-like and three luminal-like breast cancer cell lines for sphere forming efficiency, size and morphology of spheres, and expression of various proteins. Our results indicated that although tumourspheres could be cultured from cells of both subtypes, they differ in their morphology and phenotype. We also performed extensive FACS and immunohistochemistry (IHC) review of spheres formed from a select panel of basal- and luminal-like breast cancer cell lines which revealed both important patterns of correlation to be considered when interpreting sphere-data from cell lines and also potential markers of subpopulations within luminal-like spheres. Investigating the differences between adherent cell lines and tumourspheres has led to the identification of markers such as CD49f, HER2 and EpCAM which appear to have increased expression on P2 tumourspheres compared to adherent cells. These markers are of interest as potential targets. HER2 in particular is an ideal target as biotherapy and immunotherapy approaches have already been used to target this molecule. Here, we investigate the potential to target this molecule with an aim to determine if sphere forming efficiency can be altered. Our results indicate that targeting HER2 expression on tumourspheres does not alter sphere forming efficiency, and suggest that our understanding of targets on tumourspheres is still not complete.
5.2.1 Results: Phenotype of cell lines does not correlate with sphere forming capacity

5.2.1.a Flow cytometric analysis for CD24/CD44 costaining reveals three patterns of expression for breast cancer cell lines; basal, luminal, and mixed phenotypes. Previous studies have reported enriched cancer stem cell properties in subpopulations with a CD44+CD24- phenotype [68, 119] whilst others indicate this merely reflects an overall ‘basal-like’ phenotype [83]. Adherent cell lines were tested for CD44/CD24 expression in order to determine if patterns of expression were predictive of molecular subtype or sphere formation (Figure 5.1). CD44/CD24 phenotypes did not predict for sphere formation. In accordance with Fillmore [83], we found that basal-like lines had a strong CD44+CD24- phenotype, often greater than 90% of the total population. Luminal lines were heterogeneous in regards to their expression of CD44 and were uniformly CD24+. These cell lines CD24/CD44 phenotype matched with the published data [83, 84, 91, 184]. Additionally, we identified a mixed phenotype population for some cell lines (BT-20, RHB1 and SVCT) which also demonstrated mixed expression of cytokeratin markers (Communication with Dr. Chanel Smart, UQCCR, data not shown). These mixed phenotype cells also were tested for expression of other proteins (CD10, CD49f, EpCAM, HER2 and MUC1) that are normally differentially expressed between basal and luminal-like cells (Table 5.1 and Table 5.2).

5.2.1.b Cell lines with poorly defined subtypes were analysed by flow cytometry to determine a putative classification. Several cell lines used in this study have been poorly defined for their exact molecular subtype in the literature (8701 BC, PMC-42 ET, SVCT, and RHB1 in particular). In order to better classify what kind of subtype these cells might fit into, flow cytometry was used to look at a panel of proteins that are largely differentially expressed between luminal and basal-like subtypes (Table 5.2). This was accomplished using three technical replicates of staining from a representative set of matched samples. Cell line 8701 BC was negative for expression of markers of basal-like origin such as CD10; however, this line was also low for expression of luminal-like markers such as EpCAM, MUC1, and to a lesser degree HER2 (expressing some HER2 protein but not overexpressing). Cell line 8701 BC did classify as a basal-like line when using the CD24/CD44 classification scheme as previously mentioned. PMC-42-ET is a mesenchymal-like cell line derived from a patient with metastatic breast cancer [185, 186]. PMC-42 ET had a combined basal/luminal phenotype by flow. PMC-42 ET expressed the luminal marker EpCAM and had some expression
of HER2 and also was null for expression of CD10 and MUC1. Using the CD24/CD44 classification scheme, PMC-42 ET was considered to be basal. Similarly SVCT had a mixed phenotype by both flow cytometry for multiple markers and using the CD24/CD44 classification. RHB1 cells were considered to be a mixed population as well as they expressed CD10, lacked expression of luminal markers, and had a mixed phenotype by the CD24/CD44 classification.
Figure 1. Flow cytometry analysis of representative cell lines for CD24/CD44 co-staining reveals a pattern of expression for basal, luminal and mixed phenotype cells. Live-gated cells were analysed by flow cytometry for expression of CD24 and CD44. Expression patterns defining basal-like, luminal-like and mixed phenotype cell lines were found to be consistent with this classification established by Fillmore et al.
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<td>Luminal²</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>MCF7</td>
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<td>Luminal</td>
<td>Luminall</td>
<td>Luminall</td>
<td>Luminal²</td>
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<td>Yes</td>
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<tr>
<td>SK-BR-3</td>
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<td>Luminal</td>
<td>ERBB2+</td>
<td>Luminall</td>
<td>Luminal²</td>
<td>Yes/Weak</td>
<td>Grape-like</td>
</tr>
<tr>
<td>T47D</td>
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<td>Yes</td>
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<tr>
<td>ZR-75-1</td>
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<td>Luminal</td>
<td>Luminall</td>
<td>Luminall</td>
<td>Luminall²</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Table 5.1
Table 5.1. Sphere Forming Capacity (SFC) of 25 established breast epithelial cell lines does not reflect known phenotype or genotype information. SFC was assessed in cells lines and was classified as ‘-’ = no spheres observed, ‘+’ = <0.01% SFC and ‘++’ > 0.01% SFC. Published data regarding each cell line origin, phenotype, or tumourigenicity was consulted and is presented in summary in this table (Kenny et al. [155], Lacroix et al. [171], Fillmore et al. [83], Croker et al. [84], Sheridan et al. [91], Hwang-Versleus et al. [184], Neve et al. [150], and Hollestelle et al [182]). Briefly, 3D morphology refers to morphology of structures generated by each cell line when grown in laminin-rich extracellular matrix and are described as round, mass, stellate or grapelike. Tumourigenicity refers to the ability of the cells to form tumours in xenograft assays. Neve and Hollestelle (intrinsic profile) classification refers to the classification given to the cell line from independent gene expression profiling studies published by these authors using Affymetrix Genechips. Neve classification is comprised of three groups: Basal-A, Basal-B and Luminal whilst that of Hollestelle include Basal, Normal, Luminal and ErbB2, Hollestelle et al. additionally classified cell lines on the basis of protein expression by two different methods of immunohistochemical classification. The cytokeratin classification included “null” (CK8/18 low, CK19-, CK5-), “luminal (CK8/18+ and/or CK19+, CK5-)”, “basal (CK8/18 low, CK19-, CK5+)” and “combined basal luminal (CK8/18+ and or CK19+, CK5+)” categories. The four protein classifier divided the cells into “luminal” (ERBB2-, ER+), ErbB2 overexpression”, “basal” (ERBB2/ER-, CK5+ and or EGFR+) and “negative” (ERBB2/ER/CK5/EGFR-). An overall classification of luminal, luminal-ErbB2 or basal was also given by Hollestelle on the basis of these combined methods. Where we had sourced additional cell lines which were not included in the above mentioned studies and cell line phenotype information was not available (PMC42-ET, 8701BC, SVCT, RHB1), we performed flow cytometry analysis as per Table 5.2. The CD44/CD24 profile of these cells lines was also consistent with this classification as established by Fillimore et al. KPL-1 had previously been described as luminal. Additionally, HBL-100 and SVCT are considered non-malignant, having been derived from non-tumourigenic tissue. HBL-100 and MDA-MB-435 cell lines are not considered by many to be breast cancer cell lines, given evidence for male and melanoma origins respectively.

Table 5.2

<table>
<thead>
<tr>
<th>Adherent Cell Line</th>
<th>CD10</th>
<th>CD49f</th>
<th>EpCAM</th>
<th>HER2</th>
<th>CD24</th>
<th>CD44</th>
<th>MUC1</th>
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<tr>
<td>8701 BC</td>
<td>± 3</td>
<td>365 ± 16</td>
<td>8 ± 2</td>
<td>494 ± 3</td>
<td>± 28</td>
<td>± 4621 ± 31</td>
<td>± 17 ± 2.9</td>
</tr>
<tr>
<td>PMC-42 ET</td>
<td>± 3</td>
<td>522 ± 6</td>
<td>3279 ± 19</td>
<td>± 1220 ± 33</td>
<td>± 80 ± 1.7</td>
<td>± 2718 ± 1.7</td>
<td>± 7.9 ± 0.5</td>
</tr>
<tr>
<td>SVCT</td>
<td>± 2</td>
<td>2526 ± 86</td>
<td>25617 ± 568</td>
<td>± 1186 ± 22</td>
<td>± 739 ± 13</td>
<td>± 1680 ± 5.7</td>
<td>± 75 ± 3.1</td>
</tr>
<tr>
<td>RHB1</td>
<td>2285 ± 87</td>
<td>2474 ± 162</td>
<td>9 ± 10</td>
<td>1267 ± 75</td>
<td>1677 ± 114</td>
<td>9586 ± 358</td>
<td>0 ± 5</td>
</tr>
</tbody>
</table>

Table 5.2. Flow cytometry analysis of unclassified cell lines. 4 cells lines that have not been well described in the literature were assessed for their adherent phenotype using a panel of antibodies. Δ-MFI are shown +/- SEM. Additionally, RHB1 P1 spheres were also assessed for their phenotype. Overall, 8701 BC demonstrated a basal-like phenotype (high expression of CD44, low expression of CD24 and EpCAM). PMC-42 ET demonstrated a combined basal/luminal phenotype by flow cytometry for most markers (high expression of CD44 and EpCAM) and a basal-like phenotype using CD44/CD24 profile. SVCT and RHB1 cells demonstrated a mixed basal/luminal phenotype (relatively high expression of CD44 an CD24, as well as expression of EpCAM for SVCT and CD10 for RHB1). RHB1 P1 Spheres were similar to adherent cells with the exception of higher expression of CD44, and lower expression of CD24 and CD10). Yellow highlight points out the critical expression analysis for classification.
5.2.1.c No correlation between sphere forming capacity and published phenotype or genotype information of breast cancer cell lines. As discussed in chapter 4, in order to get a more comprehensive understanding of sphere formation, we tested the ability of 25 breast cancer cell lines to form spheres. Results are summarized in Table 5.1 and presented together with other phenotypic classifications given to the cell lines by other large scale studies in order to examine any possible relationships with sphere formation. Comparison to phenotype and genotype data available from other large scale studies [150, 173, 182, 187] revealed no clear correlation to sphere formation. A Fisher’s exact test was used to examine the significance of the contingency between sphere formation and subtype categorisation. Non-sphere-forming and sphere-forming cells lines fell in similar proportion amongst both the luminal-like and basal-like categories and subcategories (both Basal-A and -B categories [150] or normal, null and basal categories [182]). No correlation between sphere formation and HER2 status was observed, nor did sphere formation correlate with p53 mutation or p16 inactivation from available data [182]. Collation of data on cell line in vivo tumorigenicity (as well as our own test of tumorigenicity using immunocompromised mouse xenografts) using various mouse xenograft models [171] also failed to stratify sphere-forming from non-sphere forming cell lines as did morphological phenotypes when grown in matrigel [155].
5.2.2 Results: Examination of phenotype and morphology/physical characteristics of P1 tumourspheres from three luminal and three basal-like lines

5.2.2.a Immunohistochemical profile of spheres reflects parental cell line phenotypic expression and luminal-like spheres exhibit some molecular heterogeneity. To investigate the different sphere types (basal or luminal) at a molecular level, immunohistochemical analysis with a panel of markers was performed by members of Dr. Chanel Smart’s lab at UQCCR (Figure 5.2). This was conducted to identify heterogeneity within spheres. All the spheres generated demonstrated cells staining for Ki-67, a marker for cell proliferation. Spheres generated from three basal-like (SUM-159-PT, HBL-100 and MDA-MB-436) and three luminal-like (MCF7, KPL-1 and BT-474) cell lines exhibited exclusive basal and luminal IHC profiles respectively. Cells comprising basal-like spheres were homogenously negative for luminal markers CK19, oestrogen receptor (ER), EpCAM, MUC1 and E-cadherin and positive for basal marker EGFR, epithelial-mesenchymal transition (EMT)-marker vimentin frequently found in a subset of basal-cell lines [182] and putative stem cell marker CD44, which has been demonstrated by others to indicate basal-phenotype rather than stem-ness [83]. Lack of E-cadherin expression in the basal cell line spheres, likely accounts for the lack of cohesiveness observed in these structures – for instance MDA-MB-436 spheres were particularly non-cohesive and broke apart during preparation. These cell lines were also CK5/6 and CK14 negative (data not shown – communication with Dr. Chanel Smart, UQCCR). Comparison of spheres and adherent cells EpCAM expression by FACS did not indicate an increase in EpCAM expression for spheres compared to adherent cells, however some rare cells did express EpCAM in HBL-100 and MDA-MB-436 (Figure 5.3). Spheres generated from luminal-like cells overall demonstrated similar fidelity to the luminal phenotype: they were positive for ER, CK19, E-cadherin, EpCAM and MUC1 and negative for vimentin and EGFR. To determine whether the spheres were producing their own extracellular matrix, Period Acid Schiff (PAS) and laminin1/2 staining was performed by members of Dr. Chanel Smart’s lab, UQCCR. Basal cell line spheres showed extensive laminin staining with 70-90% immunopositivity compared to more heterogenous and intermittent staining in the luminal cell line structures (10% for BT-474 up to 60% in KPL-1) (Figure 5.2k). Interestingly, all samples demonstrated mostly cytoplasmic laminin staining and did not delineate any border surrounding the structures. The PAS stain, which stains glycogen, mucin, mucoprotein, and glycoproteins, delineated a proportion of cells comprising luminal cell spheres (blacks arrows) and was sometimes observed in an apical linear pattern in MCF7 and KPL-1 spheres (white arrows) (Figure 5.2l).
Figure 5.2 Part One
Figure 5.2 Part Two

Figure 5.2. Immunophenotyping of breast cell line spheres. Immunohistochemical analysis of indicated antigens on FFPE preparations of spheres from three basal-like and three luminal-like and cell lines. Images were taken at 200x magnification, unless where indicated by black triangle at 400x magnification. Scale bar represents 100 mm. Black arrows indicate intermittent laminin1/2+ and PAS+ cells in luminal cell spheres. White arrows indicate bright PAS staining along edge of MCF7 and KPL-1 spheres. [Work conducted by Dr. Chanel Smart and members of her lab, UQCCR.]
Figure 5.3. Representative scatter plots of EpCAM expression for three basal-like lines grown under adherent and sphere conditions. Comparison of average positivity between adherent cells or spheres did not reveal an increase in EpCAM positive cells for spheres (2-3 independent experiments). Representative data shown.
In contrast to the basal-like spheres, molecular heterogeneity was observed both between and within the luminal-like spheres for several markers. CD44 membrane expression was heterogeneous within spheres of luminal cells, although the immunopositive frequency appeared to differ considerably between cell lines (Figure 5.2i). MCF7 cells showed the highest CD44 immunopositivity, 40-50% of all cells, which tended to be localised together within the structures rather than dispersed throughout. Cells staining intensely for membrane CD44 were found next to completely negative cells. 10-20% of cells comprising BT-474 spheres were immunopositive for CD44 and often in an incomplete membranous pattern. CD44 immunopositivity was most rare in KPL-1 spheres (approximately 2-5%).

As expected BT-474 spheres demonstrated homogenous HER2 overexpression, equivalent to 3+ staining by pathological assessment, whilst MCF7 and KPL-1 cells exhibited very weak HER2 immunostaining that in a diagnostic setting would be classified at 0. This result was consistent with what was observed by flow cytometry (Figure 5.4a). MCF7 and KPL-1 cells expressed less HER2 as spheres and adherent cells compared to BT-474 cells. Additionally, at P1 by flow cytometry spheres did not demonstrate an increase in HER2 expression compared to matched adherent cells (Figure 5.4a). By IHC, ER immunostaining was also variable, the majority of MCF7 cells (80-100%) exhibiting moderate to intense (2-3+) ER staining, 40-60% of KPL-1 spheres cells exhibiting low to moderately intense staining (1-2+) and 20% of BT-474 cells exhibiting moderate ER-immunostaining.

Detection of EpCAM by IHC methods revealed high frequencies of membrane staining in the luminal-like spheres with a range of intensities spread throughout the population (Figure 5.2f), consistent with what was observed by flow cytometry (Figure 5.4b). At P1 spheres did not demonstrate an increase in EpCAM expression over matched adherent cells (Figure 4b). Basal-like cells did not demonstrate strong staining for EpCAM and as previously mentioned EpCAM was not increased for spheres over adherent cells (Figure 5.4c). MUC1 also demonstrated heterogenous staining with luminal-like spheres; with mostly diffuse cytoplasmic staining although prominent ‘apical’ or membranous staining was sometimes observed in KPL-1 and MCF7 spheres (Figure 5.2g).
Figure 5.4. Flow cytometry analysis of HER2 and EpCAM surface expression on luminal-like breast cancer cell lines. Live-gated cells were analysed by flow cytometry for Δ-median FI of HER2 (a) or EpCAM (b). Representative data of three technical replicates from one experiment. Is shown No difference between matched adherent and day 6 sphere cultures were found for either marker. (a) Δ-median FI for HER2 was found to be significantly greater for BT-474 cells (adherent or spheres) than for the other two lines (p ≤ 0.0033). (b) KPL-1 cells (adherent or spheres) were found to have a higher Δ-median FI for EpCAM than the other two cell lines (p ≤ 0.0098). Bars represent the average of 3 (BT-474) to 4 (the remaining two cell lines) independent experiments. (c) Δ-median FI for EpCAM for three basal-like lines grown using adherent or sphere-promoting conditions demonstrating no increase in expression for spheres over adherent cells. Bars represent the average of 2 (MDA-MB-436) or 3 independent experiments (the remaining cell lines). Error bars represent SEM. Significance was calculated using two-tailed unpaired t-test.
5.2.2.b Physical characteristics of three luminal-like and three basal-like spheres. We further characterised sphere formation in three basal-like (SUM-159-PT, HBL-100 and MDA-MB-436) and three luminal-like (MCF7, KPL-1 and BT-474) cell lines shown growing in adherent and sphere-promoting conditions in Figure 5.5a. Each of these cells lines was capable of at least five serial passages as spheres and sphere forming efficiency was also assessed (Chapter 4). In order to identify any morphological differences between the spheres from different cell lines, both sphere size in live bulk cultures and histopathology of H&E sections of formalin-fixed, paraffin-embedded preparation were analysed. Determining sphere size was done with close collaboration with Dr. Chanel Smart, UQCCR and preparation of H&E sections was done by members of Dr. Chanel Smart’s lab. Sphere diameters were assessed digitally in random fields (Figure 5.5b). SUM-159-PT and BT-474 tumourspheres were the smallest, averaging 54.2 μm (±18.5) and 57.3 μm (±25.6) respectively. On average, the largest structures were made by KPL-1 cells at 210.0 μm (±101.2) followed by MDA-MB-436 at 161.7 μm (±84.7). Intermediate to these were MCF7 at 117.9 μm (±42.4) and HB-L100 at 92.4 μm (±47.0). These results indicate no correlation between sphere size and luminal or basal phenotypes at first passage.

5.2.2.c Distinct sphere morphologies in basal and luminal breast cancer cell lines. Examination of H&E sections of the different spheres revealed multiple differences between basal-like and luminal-like spheres (Figure 5.5a). Both HBL-100 and SUM-159-PT spheres are solid structures with an irregular edge, comprised of cohesive but loosely packed cells. MDA-MB-436 spheres appeared to completely disintegrate upon histological preparation. In contrast to the basal-like cell lines, MCF7, KPL-1 and BT-474 spheres were comprised of tightly packed cohesive cells which formed within a well-defined edge. Whilst BT-474 spheres were always spherical solid masses, a proportion of KPL-1 and MCF7 spheres were hollow. KPL-1 structures were consistently of an irregular shape and contained inner cleft-like lumina reminiscent of ductal carcinoma in situ (DCIS). MCF7 spheres exhibited ranging morphologies, including solid masses, single or multiple cell layers enclosing lumina or asymmetrical structures exhibiting a mass on one side of the structure with an enclosed lumen. Mitoses and apoptotic cells were prominent in spheres from all six cell lines and spread throughout the structures. Cells undergoing squamous differentiation were sometimes observed in the periphery of KPL-1 spheres.
Figure 5.5. Morphologies and sphere-forming characteristics of basal-like and luminal-like lines. (a) Light microscope images of cell lines grown in adherent and tumoursphere-promoting conditions 7 days after initial seeding [40x magnification]. H&E analysis of sections of formalin-fixed, paraffin embedded spheres. Arrows indicate wide-lumina observed within MCF7 spheres and cleft-like luminal observed within KPL1 spheres. Scale bars represent 100 μm. (b) Sphere sizes were assessed 7-days after seeding. The experiment was repeated twice and representative results are graphically presented. Bars represent the average diameter of the long-axis of the spheres, error bars represent SEM. A two-tailed unpaired t-test demonstrated no difference in sphere size between the luminal and basal-like lines tested. [Work done in collaboration with Dr Chanel Smart and members of her lab, UQCCR]
5.2.2.d Luminal cell lines exhibit heterogenous MUC1 surface expression with increased expression exhibited by spheres. The heterogeneity observed within the luminal-like spheres for MUC1, EpCAM and HER2 prompted further investigation by flow cytometry, which was consistent with immunohistochemistry results. In order to determine whether the frequency of these molecules was increased in spheres compared to the adherent population, flow cytometry was performed on matched sphere and adherent cultures. Only MUC1 was shown to demonstrate increased expression on the cell surface of all three luminal cell lines cultured as tumourspheres compared to adherent cultures in terms of both median fluorescence intensity (Figure 5.6a) and percentage positivity (Figure 5.6b). Figure 5.6c shows representative MUC1 histograms for the three luminal cell lines. In order to determine whether heterogeneity in MUC1 expression delineated different subpopulations in terms of their sphere forming ability, we assessed sphere forming efficiency in MUC1 high and low expressing cells (top 15% and bottom 15% of MUC1 expression respectively) sorted from the parental/adherent populations of MCF7 and KPL-1 cells (Figure 5.6d). No difference was found in the sphere forming efficiency in MCF7 MUC1\textsuperscript{hi} (0.9 ± 0.5%) and MUC1\textsuperscript{lo} cells (0.8 ± 0.4%) (Figure 5.6d). KPL-1 MUC1\textsuperscript{hi} cells (7.3 ± 4.4%) did demonstrate increased sphere forming efficiency compared to MUC1\textsuperscript{lo} cells (5.3 ± 2.5%). In order to determine if MUC1 high and low expressing cells retain their high or low phenotype over serial passage as spheres, MUC1 high and low cells were sorted from adherent cells, grown as tumourspheres, and assessed for MUC1 expression at P1 (day 5) or P3 (day 15). MCF7 P3 tumourspheres sorted for MUC1\textsuperscript{hi} expressed MUC1 significantly greater than adherent cells or P3 MUC1\textsuperscript{lo} cells, but expression was decreased compared to expression levels at P1 (Figure 5.6e). KPL-1 P3 MUC1\textsuperscript{hi} cells also demonstrated a decrease in expression from P1 and were not significantly different to adherent cells or P3 MUC1\textsuperscript{lo} cells.
Figure 5.6. Flow cytometry analysis of MUC1 surface expression on luminal-like breast cancer cell lines grown in matched adherent and sphere-promoting conditions. (a) Live-gated cells were analysed by flow cytometry for Δ-median fluorescent intensity (FI) of MUC1. Differences between adherent and sphere cultures 6 days after seeding were shown to be significant for KPL-1 (p=0.0006), MCF7 (p=0.0084), and BT-474 (p = 0.0069). (b) Frequency of MUC1-positivity in matched adherent and sphere cultures of luminal-like cell lines. Differences were shown to be statistically significant for KPL-1 (p = 0.0003), MCF7 (p = 0.0016), and BT-474 (p = 0.0067). For both (a) and (b) bars represent the average of n = 3-5 independent experiments. Significance was calculated using two-tailed unpaired t-test. (c) Representative FACS histogram plots for the luminal-like cell lines demonstrating surface expression of MUC1 on spheres (blue line) and adherent cultures (red line). Green and orange lines represent the unstained adherent and sphere controls respectively. (d) SFE for sorted MCF7 and KPL-1 MUC1<sup>hi</sup> and MUC1<sup>lo</sup> cells at P1 showing a significant increase in SFE for KPL-1 MUC1<sup>hi</sup> over MUC1<sup>lo</sup> cells (p = 0.0075). (e) Δ-median FI for MUC1 expression for P1 and P3 MUC1<sup>hi</sup> and MUC1<sup>lo</sup> cells sorted from adherent (ADH) parental cells. P1 MUC1<sup>hi</sup> cells were found to have increased expression of MUC1 compared to parental cells and MUC1<sup>lo</sup> cells (p=0.0001 for MCF7 cells and p=0.0074 for KPL-1 cells). P3 MUC1<sup>hi</sup> and MUC1<sup>lo</sup> cells were found to decrease MUC1 expression for both cell lines. P3 MCF7 MUC1<sup>hi</sup> cells demonstrated increased expression of MUC1 over adherent cells and P3 MUC1<sup>lo</sup> cells (p ≤ 0.0031), while P3 KPL-1 MUC1<sup>hi</sup> cells were found to not have significantly different expression of MUC1 compared to adherent cells, or P1/P3 MUC1<sup>lo</sup> cells. Error bars represent SEM.
5.2.3 Results: Examination of phenotypic characteristics of P2 tumourspheres between luminal and basal-like lines

5.2.3.a The CD44+CD24- phenotype is not enriched in tumoursphere culture. CD44/CD24 phenotype in P2 tumoursphere cultures for two basal-like lines (SUM-159-PT and HBL-100) and two luminal lines (MCF7 and T47D) was compared to matched adherent cells (Figure 5.7). Cells grown as tumourspheres were found to express a similar pattern of CD44/CD24 expression as the parental/adherent line. One exception to this was T47D which was found to dramatically lose expression of CD44 upon culture as tumourspheres. Tumourspheres from basal-like lines were strongly CD44+CD24- while tumourspheres from luminal lines were CD24+ and had variable expression of CD44. Overall, CD44 expression was found to decrease in culture as tumourspheres (Figure 5.7b). For these four lines tested the decrease in expression for CD44 was found to be significant with p values below 0.0002, **

5.2.3.b CD49f expression increases on tumourspheres compared to matched adherent cells. Similar to what has been reported for MCF7 tumourspheres by Cariati et al., we found an increase in expression of cell surface CD49f for tumourspheres compared to adherent cells. The expression of CD49f was not found to be significantly different between basal (SUM-159-PT, HBL-100, MDA-MB-436) and luminal cell lines (KPL-1, MCF7, T47D, Hs578T and ZR-75-1) when testing adherent cultures (Figure 5.8a). Comparing matched adherent culture and P2 spheres an increase in CD49f was found for tumourspheres for both the basal and luminal lines tested (P≤0.0008, ***) (Figure 5.8b).

5.2.3.c HER2 and EpCAM expression is higher in cells of luminal origin than basal-like origin and increases upon culture of luminal lines in tumoursphere-promoting conditions. As previously mentioned adherent luminal lines were found to express higher levels of HER2 and EpCAM compared to basal-like lines. Comparing the mean fluorescent intensity for two luminal lines (MCF7 and T47D) to two basal-line lines (SUM-159-PT and HBL-100) grown under adherent conditions demonstrates that luminal lines express more HER2 and EpCAM (p ≤ 0.0001, ***) (Figure 5.9a). The two luminal lines tested demonstrated increased expression of HER2 and EpCAM for P2 tumourspheres compared to matched adherent cells (p ≤ 0.011, * and 0.004, ** respectively) (Figure 5.9b). Conversely, the two basal-like lines demonstrated decreased expression of HER2 and no change
or decrease in expression for EpCAM (noted to already be quite low) for tumourspheres at P2 compared to adherent cells (Figure 5.9b).

Figure 5.7. CD44/CD24 phenotype of matched adherent and P2 tumourspheres. (a) Representative dot plots of the expression of CD44 (vertical axis) and CD24 (horizontal) are shown for cells within a PI gate for live cells. Plots are representative from at least two separate experiments. (b) Differences in expression levels (mean fluorescent intensity) for CD44 between adherent and P2 tumourspheres was evaluated by flow cytometry. The four cell lines tested demonstrated decreases in CD44 expression for tumourspheres with P values below 0.0002, *** (two-tailed t-test). Plots are from 3 technical replicates from representative data of at least two separate experiments.
CD49f expression increases on P2 tumourspheres compared to adherent cells.

Live-gated cells were evaluated for expression of CD49f by flow cytometry, and values are expressed as representative Δ-mean fluorescent intensities. Values here represent the mean of three replicates and are representative of one experiment from at least two separate experiments. Error bars represent SEM. (a) Adherent cells representative CD49f expression for five luminal lines (KPL-1, MCF7, T-47D, Hs578T and ZR-75-1) and three basal lines (SUM-159-PT, HBL-100 and MDA-MB-436). No statistical difference between basal or luminal lines was found using a t-test. (b) Comparison between matched adherent cultures and P2 tumourspheres indicates that P2 tumourspheres demonstrate increased expression of CD49f for both luminal and basal-like cell lines, in all cases p ≤ 0.0008, ***.
Figure 5.9. HER2 and EpCAM phenotype of adherent cells and P2 tumourspheres. Cells were evaluated by flow cytometry for expression of HER2 and EpCAM, and values are expressed as Δ-mean fluorescent intensity (FI). Data is representative data (n=3 technical replicates) from one out of two experiments. (a) Adherent cell expression of HER2 and EpCAM. Luminal cells tested expressed significantly more HER2 and EpCAM than basal-like cells (p ≤ 0.0001, ***) using a t-test. (b) HER2 and EpCAM expression differences between adherent cells and P2 tumourspheres. For HER2, the increase in expression for luminal spheres was found to be significant with all p values below 0.011, *. The decrease in HER2 was found to be significant for basal-like lines with all p values below 0.0001, ***. For EpCAM, the increase in expression for luminal lines was significant with all p values below 0.004, **. The decrease in EpCAM for basal lines was not found to be significant for HBL-100 cells and significant for SUM-159-PT (p value = 0.0014) Note that these lines express low levels of EpCAM regardless. Error bars represent SEM.
5.2.3.d HER2 cell surface expression decreased by treatment with trastuzumab with no changes in SFE, cell cycle or viability of cells. As a result of the findings that HER2 expression was increased for P2 tumourspheres for MCF7 and T47D cell lines compared to adherent cells and the concept that rare breast cancer stem cells might be regulated by HER2 expressing cells, we investigated the effects of a monoclonal antibody against HER2 (trastuzumab or Herceptin™) on a variety of biological parameters. The levels of HER2 cell surface expression as assessed by flow cytometry on day 5 after two passages in the presence of the antibody were found to decrease for both adherent cells and for P2 tumourspheres when treated with 20 μg/mL trastuzumab compared to no treatment (p ≤ 0.0001, ***). Additionally, no difference was noted for treatment with a control antibody at the same concentration - rituximab (recognises CD20). No difference was noted in the viability of cells treated compared to non-treated as assessed by PI staining (data not shown). This is in agreement with reports showing that trastuzumab treatment could reduce HER2 surface expression in HER2 expressing cell lines but not induce apoptosis [85]. No further decrease in cell surface HER2 expression was observed by incubating MCF7 tumourspheres with 40 μg/mL or by incubating T47D tumourspheres with 40 or 80 μg/mL (data not shown). Treatment with trastuzumab did not result in any changes to the cell cycle. At both 24 hours and 72 hours after initial treatment with trastuzumab both non-treated and treated cells showed similar levels of cells in each of the stages of the cell cycle (data not shown). The effect of trastuzumab treatment on sphere forming efficiency was then assessed at day 5 for these two cell lines at P1, P2 and P6 and starting with 5000 or 10000 single cells per well. SFE was found to not be affected by treatment with either trastuzumab or rituximab for either cell line (Figure 5.10b and 10c). MCF7 and T47D were determined to be trastuzumab resistant cell lines in vitro. This was demonstrated by comparing the growth of control adherent cells to the growth of cells co-cultured with either trastuzumab or rituximab. For MCF7 and T47D cell lines there was no difference in the growth of these cells over serial passage with or without treatment (Figure 5.11a). BT-474 cells were found to be sensitive to treatment with trastuzumab but not to treatment with rituximab. Cell culture in the presence of trastuzumab could not continue past passage 4 as too few cells were left for culture (Figure 5.11a). Sphere forming efficiency at P1 was also tested for BT-474 cells in the presence of trastuzumab or rituximab. A non-significant decrease in SFE was found for co-culturing BT-474 cells with trastuzumab (Figure 5.11b).
Figure 5.10. Trastuzumab treatment and spheres. (a) Trastuzumab (Herceptin) treatment was found to decrease the cell surface expression of HER2 as assessed by flow cytometry for both adherent cells and tumour spheres at P2. Rituximab treatment was not found to have an effect on HER2 expression. \( P \leq 0.0001 \), ***, Representative results are displayed (multiple experiments were done but not always with the rituximab control). (b) For MCF7 and (c) T47-D, neither Herceptin nor rituximab was found to have an effect on SFE when combined data from P1, P2 and P6 (starting from 5000 or 1000 single cells per well, 15 wells) was assessed. P1 SFE efficiency was conducted at least twice with similar results. Representative results are shown. Columns are means and error bars represent SEM.
Figure 5.11. Trastuzumab treatment and *in vitro* adherent growth and BT474 SFE. (a) MCF7 and T47D cell lines growth was not affected by co-cultured with either trastuzumab or rituximab at 20 μg/mL. Done in triplicate over serial culture in T-25 tissue culture flasks. BT-474 cells were found to be sensitive to treatment with trastuzumab but not to treatment with rituximab. (b) BT-474 sphere forming efficiency at P1 (using 5000 and 1000 single cells per well) was tested in the presence of trastuzumab or rituximab. A non-significant decrease in SFE was found for co-culturing BT-474 cells with trastuzumab. Columns are means and error bars represent SEM.
5.3 Discussion

5.3.1 Phenotype of cell line does not correlate with sphere forming capacity and morphological and phenotypic characteristics of P1 tumourspheres. Our screening of breast cancer cell lines sphere forming capacity reveals that cell lines of multiple subtypes have the capacity to form spheres. Sphere formation was detected across the basal/luminal distinction and both sphere formers and non-formers alike were observed in cell lines of basal-A, basal-B and luminal gene expression profiles. This suggests that gene expression profile cannot predict sphere-forming capacity. It also suggests that cells capable of forming a sphere could come from distinct backgrounds – simply put either basal or luminal. Furthermore, spheres were not formed from all cells lines tested. These findings might mean that spheres represent progenitor cells of the lineage that the cell line originates from and not true stem cells, and that not all cell lines can form spheres under the conditions that we have used.

Notwithstanding that our summary of in vivo tumorigenicity for each cell line is comprised of multiple different studies and methods (reviewed in [173, 187], there is a remarkable lack of correlation between sphere forming capacity and in vivo tumorigenicity. For instance, some cell lines that are relatively poor at tumorigenicity did form spheres (Hs578T and HBL-100). The fact that sphere formation was not correlated with tumorigenicity is to some extent counterintuitive given the evidence to date that breast cancer cells grown as spheres are more tumourigenic in vivo than their adherent counterpart [77] which has lead to the understanding of a relationship between ability to form spheres in vitro and in vivo tumorigenicity. That many non-sphere forming cells can produce tumours in vivo may suggest the existence of another type of tumour-initiating cell which does not produce spheres in vitro culture. Conversely, the sphere-forming proficiency of the non-tumourigenic HBL-100 cell line indicates that; (i) sphere formation is not directly related to tumorigenicity; and/or (ii) it does not perfectly correlate with the presence of breast cancer stem cells; or further (iii) that the biological significance of in vitro sphere formation is more complicated than has come to be accepted. This discrepancy in HBL-100 cells may add to the intrigue surrounding this cell line of somewhat unclear but genotypically male origin [188]. On the other hand, the finding that sphere forming capacity is not correlated to tumorigenicity is perhaps not surprising given that normal breast epithelial cells can form spheres in vitro. Sphere formation appears to be a characteristic of cell lines that does not depend on whether that cell line is a tumour cell line or a normal cell line. Cell lines can continually be passaged in culture and
therefore maybe considered to be stem cell-like or have cells with the similar characteristics as stem cells. Many cell lines with previously demonstrated tumorigenicity \textit{in vivo} did not form spheres in our culture conditions. Similarly, the ability to form 3D structures in matrigel and the morphologies of these structures also did not correlate with sphere formation in our analysis. Again this is not surprising as normal breast cells can also grow in these conditions.

There is clear correlation between the adherent and sphere morphologies of the cell lines we tested. Basal-like and luminal-like spheres demonstrate similar size and SFE but have a myriad of morphological and phenotypic differences. However, the overall resemblance of spheres to tumours \textit{in vivo} is striking and prompts the suggestion that culture in these conditions may more faithfully model the \textit{in vivo} scenario. Indeed the importance of 3D modelling in \textit{in vitro} breast epithelial assays utilising extracellular matrix is well established [189, 190]. When grown in laminin-rich matrix, normal and malignant breast epithelial cells demonstrate dramatic morphological and molecular differences compared to 2D cultures and importantly show crucial differences in apoptotic sensitivity in response to chemotherapeutics [191]. Differences between 2D and 3D cultures are thought be the result of a combination of factors including, the provision of extracellular signals by the laminin rich matrix. It is possible that sphere formation assays are a similar 3D model, albeit matrix-free. There is strong evidence presented here and elsewhere that under certain conditions tumourspheres/mammospheres secrete their own matrix [192, 193] and indeed, mammospheres from normal human breast epithelial cells secrete laminin and embryonic matrix proteins tenascin and decorin [33]. Importantly, the cell-cell contact that spheres display should be considerably closer to the contact that cells have in the \textit{in vivo} microenvironment and would be quite different to those contacts cells have when grown attached to tissue culture plastic. Others have shown that the this type of cell-cell contact as well as support from extracellular matrices is important in providing physical and biochemical cues and allows cells to self-organise transcriptionally and architecturally [190, 194, 195]. Research has shown that besides the morphological differences between cancer cells grown in 2D and 3D systems, there are many biochemical differences, gene expression differences, and differences in the response to chemotherapy [155, 190]. Hence, we have been interested in characterising the differences between cell lines grown as tumourspheres and grown in standard 2D tissue culture plastic.

Systematic review of breast cancer growth in 3D laminin rich matrix revealed distinct morphological phenotypes described as mass, grape-like, round and stellate and it has been proposed that these morphologies correlate with cell line gene expression profile [155].
Interestingly, out of eight lines that form spheres in our conditions and have a described 3D morphology by Kenny et al., the stellate morphology was described for the cells that gene cluster with basal-B and the mass or grape-like morphology was described for cells that gene cluster with luminal lines. Luminal lines MCF7 and BT-474 display a mass phenotype as 3D morphology, and together with KPL-1 have a solid cohesive structure as tumourspheres. The morphology of this mass phenotype is strikingly similar to the morphology of spheres grown under NSA tumoursphere-promoting culture conditions. MDA-MB-436 was shown to have a stellate-structure as 3D morphology, which, along with HBL-100 and SUM-159-PT, in tumourspheres were non-cohesive loose structures. The stellate morphology under 3D culture conditions was likewise very similar to the conditions of growth of tumourspheres under NSA culture. It therefore appears that there is a similarity between tumoursphere conditions without exogenous matrigel and 3D structures that form in matrigel conditions. It is furthermore conceivable that culture conditions that provide extracellular matrices do so in excess of what is physiologically needed and hence are not faithfully modelling the in vivo situation. We propose, given the evidence that spheres generate their own extracellular matrix that the tumoursphere-promoting culture condition is in addition to being a model for examining LTP self-renewal is a system that more accurately models in vivo tumours in vitro.

Breast epithelial cells (non-malignant, malignant and established cell lines) grown as spheres have been shown to be functionally different to their respective control populations. Whilst this is usually interpreted as differences due to the selection or enrichment of a particular cell type, it is also possible that some of these differences are simply due to responses of the cells being grown in serum-free suspension culture. We already know that significant gene expression changes occur in cell when grown in 3D compared to 2D conditions; indeed we also know that the gene expression profile of breast cancer cell line spheres is different to their 2D counterparts [78]. It may be possible that some of these differences, induced by the different culture methods, prime the cells for a different gene expression. Kok and colleagues attempted to address this by including controls of 2D adherent cells cultured without serum (as for spheres) and found that serum withdrawal did not affect the expression of their markers of interest (ER and PR) [78]; however, it is still possible that other genes are affected by the media condition, and further that other differences between monolayer culture and suspension culture are not accounted for. It is possible that sphere culture is a good compromise between 2D monolayer culture and 3D matrigel culture. Sphere culture allows cells to grow in a more in vivo like structure yet remain
without the restriction of a fixed exogenous matrix, which may itself be providing cues excessive of the in vivo scenario.

Overall sphere IHC phenotype was representative of the basal-like or luminal-like parental cell phenotype. Spheres derived from basal-like cells displayed basal markers and a lack of luminal markers and vice versa for spheres derived from luminal-like lines. Spheres of basal-like origin were homogenous in their expression of markers while some heterogeneity was found for spheres of luminal-like origin. This could indicate that spheres are composed mostly of cells that have similar characteristics to the parental/differentiated cell line – luminal or basal. Rare LTP cells that should reside within spheres might be missed by this method and hence not phenotyped. Alternatively, these findings might indicate that spheres are composed of TICs that do not have a specific different phenotype to other cells in the sphere that are not TICs. More research needs to be conducted to determine this. However, heterogeneity within spheres calls into question the biological significance of each phenotypic population of cells. Recently, one report has shown that ER positivity is reduced in luminal mammospheres compared to adherent cells [78]. Our finding that not all cells in luminal-like sphere express ER is an interesting example of heterogeneity within spheres. Other heterogeneous markers identified in this study were EpCAM, HER2, CD44/CD24, and MUC1.

EpCAM is a glycoprotein expressed on epithelial cells and tumours [196]. EpCAM has been used to further isolate putative breast cancer stem cells from primary tumours with a more tumorigenic potential from the CD44+CD24−/low population, resulting in a 50-fold enrichment for the ability to form tumours compared to EpCAM− counterpart [68]. In cell lines, EpCAM has also been used to enrich for cells with a more tumorigenic potential with as few as 100 Sum-159 CD44+CD24−/low EpCAM+ cells forming tumours in mice, whereas unsorted or CD44+CD24−/low EpCAM− cells could not [83]. EpCAM and CD24 have been previously shown to co-express in luminal lines and not in basal lines, despite the observation that EpCAM+ cells could be isolated in varying frequencies from basal-like, luminal and mixed cell lines and that its expression correlated to sphere formation and tumour-initiating capacity [83]. As such EpCAM expression was studied within spheres compared to adherent cells. The present study shows that EpCAM is expressed in luminal lines that are characterised as CD24+. Our IHC results indicate an absence of EpCAM expression within basal-like spheres although our flow data did suggest an extremely rare EpCAM+ positive population in HBL-100 and MDA-MB-436 but not SUM-159-PT cells. Although these populations were found to be more rare than those described by Fillmore et al, our
The product of the \textit{HER2/neu (ErbB2)} oncogene, a member of the epidermal growth factor receptor tyrosine kinase family, is overexpressed in some subtypes of breast cancer and is associated with a poorer prognosis \cite{95, 96}. The role that HER2 overexpression plays in regulating breast cancer stem cells has been recently investigated \cite{85}. The work by Korkaya \textit{et al.} demonstrated that the overexpression of HER2 was capable of increasing the number aldeflour-positive cells, thought to be part of the cancer stem cell pool, and also resulted in increased invasiveness of these cells. Our results demonstrate that HER2 expression was virtually absent from the three basal-like lines we tested and expressed by luminal-like lines. HER2 expression was found to be highest and most uniform for BT-474 cell line. This pattern of expression did not change between cells cultured as spheres at P1 and matched adherents. Immunohistochemically there was foci of stronger HER2 expression in spheres from KPL-1 and MCF7 indicating the heterogenous expression of this marker and perhaps differential expression in cells sustaining proliferation of spheres \textit{vs.} not.

CD24 is a glycosylated mucin-like cell surface protein involved in cell adhesion and signalling that has been linked to malignancies including epithelial ovarian cancer and breast cancer \cite{197, 198}. In breast cancer CD24 has been associated with a poor prognosis and with metastasis through the role of CD24 being an alternative ligand of P-selectin, an adhesion molecule on activated endothelial cells and platelets \cite{198}. We found that CD24 expression was associated with luminal cells. CD24 might be a potential target for breast cancer and potentially for luminal TICs/progenitor cells as CD24 cross-linking has been found to inhibit migration and induce apoptosis in MCF7 cells \cite{199}. CD44 is a cell surface receptor for hyaluronic acid involved in biological processes such as cell-cell and cell-extracellular matrix adhesion, migration, and wound healing as well as playing a role in tumour pathology such as tumour differentiation, invasion and metastasis of cancer cells. CD44 expression has been associated with upregulation of genes that have links to cancer such as TGF-\(\beta\), genes associated with WNT signalling, cell adhesion and chemokines \cite{200, 201}. Many studies (referenced elsewhere in this thesis) have examined the expression of CD44/CD24 within breast cancer cell lines and patient tumour samples. The CD44\textsuperscript{+}CD24\textsuperscript{−} phenotype has largely been used to indicate the presence of BCSCs or
TICs. However, recently other researchers have called into question the reliance on this marker for identifying breast cancer stem cells. Examination of cell lines in our panel indicates that CD44/CD24 co-staining reveals three subtype profiles (basal, luminal and mixed) similar to that found by Fillmore et al. This mixed phenotype was found for three cell lines BT-20, SVCT, and RHB1. Each of these three cell lines was identified as having a mixed basal/luminal phenotype either by Hollestelle et al. for BT-20 or by flow cytometry for a panel of markers conducted by us (Figure 5.1 and Table 5.1 and Table 5.2). This adds further weight to the concept that CD44/CD24 staining does delineate three distinct phenotypes of breast cancer cells. The molecular heterogeneity seen in CD44 both by FACS and immunostaining reveals a correlation with basal-ness rather than sphere forming capacity. However, it would be instructive to further characterise the rare CD44 immunopositive cells seen in spheres from luminal lines, perhaps in combination with other markers such as EpCAM, for both SFE and tumorigenicity.

MUC1 is a Type I membrane glycoprotein of the mucin family normally expressed at the apical border of healthy epithelial cells [115, 202-204]. In cancerous tissue, MUC1 protein expression is aberrantly expressed on multiple cell surfaces on as much as 75% of human solid tumours and greater than 90% of human breast cancer and subsequent metastases [205-208]. Increasing evidence has demonstrated a role for MUC1 as an oncoprotein, including demonstrations of a role in promoting increased cell growth rate [209], in anti-apoptosis [207], and in promoting anchorage independent cell growth [210]. Additionally, a low molecular weight MUC1 cleavage product, MUC1*, that remains membrane bound has recently been shown to be a determinant of trastuzumab resistance in HER2+ breast cancer [211] and functions as a growth factor receptor [212, 213]. MUC1 has been proposed as a target for immunotherapy of breast cancer with some clinical trials exploring various MUC1-based vaccines having been conducted [214-216]. Tumour-associated alterations of MUC1 such as hypoglycosylation, increased sialylation, and altered carbohydrate core-type expression are responsible for the antigenicity of MUC1 and hence its suitability as a target for immunotherapy [115, 135, 217]. MUC1 expression has recently been reported to be expressed on the side population cells from MCF7 mammosphere cultures demonstrating for the first time that MUC1 is not only expressed on mature breast cancer cells but also on cells that have some of the functional characteristics of cancer stem/progenitor cells [130]. Immunotherapy approaches that target breast cancer stem/progenitor cells has the potential to eliminate minimal residual disease and may lead to more meaningful clinical remissions [108]. Further characterisation of MUC1 expression on stem/progenitor cells will be instrumental for the future application of MUC1-based tumour vaccines.
Importantly, our results show that MUC1 expression is increased on luminal tumourspheres compared to adherent cells and are heterogeneously expressed within spheres. This suggests that at the least tumourspheres or cells with tumourspheres may be enriched with anti-apoptotic or pro-growth properties. By both immunohistochemistry and flow cytometry MUC1 was found to be highly expressed by most KPL-1 cells within tumourspheres. MCF7 cells displayed a more heterogeneous expression of MUC1 as assessed by immunohistochemistry and flow cytometry. BT-474 displayed good staining for MUC1 by immunohistochemistry but not by flow cytometry, perhaps due to differences in the antibodies used to assess MUC1 positivity. Even so BT-474 spheres demonstrated increased MUC1 expression over adherent cells. Previous studies have shown that MUC1 is a co-receptor for HER2, EGFR, and FGFR3 [208, 218]. It is tempting to speculate that MUC1 is modulating the signalling through these molecules in a subpopulation of cells to promote tumoursphere self-renewal or tumourigenesis.

Our results sorting for MUC1\textsuperscript{hi} and MUC1\textsuperscript{lo} cells demonstrate that MUC1 does enrich for SFE in KPL-1 cells but does not solely account for sphere formation as MUC1\textsuperscript{lo} cells are also capable of sphere formation. MUC1 expression from both cell lines within cells that were sorted for high and low expression was found to return to a baseline value over time. In other words MUC1\textsuperscript{hi} cells were found to lose expression of MUC1 as a population and have a more uniform expression similar to cells before sorting. This might indicate that MUC1 is not a direct marker of BCSCs. Indeed the expression of MUC1 being expressed by a large portion of cells within the population does not fit with the notion of BCSC markers should only be present on a rare portion of cells. Perhaps MUC1 expression is increased on spheres as a result of the culture conditions being changed relative to adherent cells rather than as an enrichment in cancer stem cells. Future studies will need to address the co-expression of MUC1 and other cancer stem cell markers on breast cancer stem cells, determine the expression of MUC1* on sub-populations of cells in tumourspheres, and go further to demonstrate the potential usefulness of MUC1-based therapy to disrupt the cancer stem cell compartment.

5.3.2 Discussion: Phenotype of P2 tumourspheres compared to adherent cells. Examination of P2 tumourspheres was conducted to assess if longer time in culture would change the expression of different cell surface markers. Tumourspheres from two luminal lines (MCF7 and T47D) and two basal lines (SUM-159-PT and HBL-100) were assessed compared to adherent cells to see if the CD44\textsuperscript{+}CD24\textsuperscript{−} phenotype was affected by culture in tumoursphere-
promoting conditions at P2. The most intriguing difference found was that luminal tumourspheres expressed similar levels of CD24 as their adherent counterparts but reduced expression of CD44. Indeed, the same finding of reduced expression of CD44 was also found for the two basal lines investigated as well. Interestingly, a recent report by Grimshaw et al. has demonstrated the presence of a CD44⁺CD24⁻ cell population in a pleural effusion sample that was cultured as tumourspheres as being highly tumourigenic. These findings indicate that the reliance on the CD44⁺CD24⁻ phenotype as being the gold standard for identification of BCSCs is wrong and that our understanding of BCSCs needs to improve. Perhaps TICs from difference phenotypes of cancer (basal/luminal) have a different phenotype than has been previously espoused. We further characterised additional markers at P2 to determine what differences in cell surface marker expression could be found between adherent cells and P2 tumourspheres.

CD49f (α-6 integrin) in association with CD104 (β-4 integrin) is expressed on normal epithelial cells and is involved in anchoring the epithelium to the underlying basement membrane [219]. CD49f has been used extensively to isolate and characterise normal mammary epithelial stem cells and has been used in combination with the CD29⁺ and CD24low phenotype to generate mammary structures from a single cell [10, 31]. In tumours, α6β4 integrin has been linked to anchorage-independent growth, sustaining tumour survival and impeding apoptosis in a vascular endothelial growth factor-dependent manner [220]. The α6β4 integrin expression has been associated with several carcinomas and has also been linked in tumours to migration and invasion [221]. These attributes are thought to be a result of a switch from the adhesive function of CD49f to a signalling component during the progression from normal tissue to invasive carcinoma [221, 222]. More recently, CD49f has been investigated in mammospheres-derived cells from the MCF7 cell line. CD49f was found to have increased expression on mammospheres and was necessary for the sphere-forming capacity of these cells in vitro and the tumorigenicity of these mammospheres in vivo [77]. We found no difference in the expression levels of CD49f between basal and luminal lines. The present study shows the increase of CD49f on a number of breast cancer cell lines when the cells are grown as tumourspheres, and this occurred regardless of the subtype of the cell line. This suggests that CD49f might play a role in tumoursphere formation, adhesion of cells within tumourspheres or even signalling that is different than the role played in monolayer culture. Additionally, it is also possible that CD49f might not be expressed by all BCSCs grown as mammospheres and hence might not be a universal marker of BCSCs – this needs to be further explored in future research. Regardless, these results confirm what has been shown previously by Cariati et al. for the MCF7 cell line and further demonstrate that this marker
is also found increased in other cell lines cultured as tumourspheres. Additionally, these findings suggest that cells that compose tumourspheres might be enriched in cells that are more tumourigenic, more resistant to apoptosis, and more invasive. All of these are characteristics of BCSCs. Importantly, CD49f expression has been linked to invasive characteristics of tumours and to the stem-cell like phenotype of tumours and hence could be a suitable target for immunotherapy to eliminate the tumour-initiating cells of a carcinoma.

An increase in EpCAM expression was noted for P2 tumourspheres compared to adherent cells. This only occurred for luminal cells as basal-like cells were found to have no change in expression of EpCAM or a slight decrease in the expression of EpCAM following culture as tumourspheres. Basal-like cells as previously pointed out have low EpCAM expression to begin with. Previous reports have demonstrated that tumourspheres from basal-like lines (in particular SUM-159-PT was used) contain a population of cells that express some amount of EpCAM assessed as a small population of cells expressing EpCAM just above the negative population [83]. Assessing histograms of our basal-like cell lines for EpCAM expression does show some rare cells expressing EpCAM above the greater population of cells, but this is quite rare, is not affected by culture as tumourspheres compared to adherent cells, and was not found for SUM-159-PT (see Figures 5.3 and 5.9). Instead, we found that luminal lines at P2 did increase the expression of EpCAM. These results together with our results for P1 luminal tumourspheres expressing EpCAM suggest that EpCAM could be a potential marker for TICs of the luminal lineage.

The current study demonstrated that HER2 is expressed at higher levels on cells within tumourspheres from luminal lines at P2 compared to adherent cells. The two cell lines used for this study were T47D and MCF7, and they have been reported to have low HER2 expression (compared to clinically HER2+ tumours) [223-226]. Despite this, these cell lines do express HER2 on the cell surface and in fact do so higher than the basal lines that we tested (SUM-159-PT and HBL-100). HER2 expression was found to increase only on tumourspheres derived from cell lines of luminal origin, and in fact HER2 expression significantly decreased for basal-like lines. This could indicate that HER2 might play a role only in some cancer stem cells. As previously mentioned, the role that HER2 overexpression plays in regulating BCSCs has been recently investigated and as such HER2 might be an important target for future trials aimed at eliminating BCSCs [85]. Importantly, Korkaya et al. have shown that increased expression of HER2 results in an increase in sphere formation. HER2 is associated with aggressive and
metastatic disease [96]. As such, the increased expression on tumourspheres may indicate the increased propensity of these cells to metastasize. HER2 expression on tumourspheres is also interesting in light of the potential to target this protein by biotherapy with trastuzumab or through immunotherapy utilising a dendritic cell-based approach as has been previously reported to target HER2 overexpressing cells in a mouse model [227]. As a result of these findings we were interested in investigating the response of sphere formation in cells to trastuzumab treatment to see if antagonists against HER2 might disrupt self-renewal of spheres. Single-agent trastuzumab response rates range from 12 to 34% for metastatic breast cancer and with improvements in survival rates for HER2-overexpressing patients treated in the adjuvant setting [228]. Despite these successes, there is a subset of patients who either do not respond to trastuzumab treatment or acquire resistance. Trastuzumab is thought to have several mechanisms for its mode of action [229]. One mode of action is through downregulation of cell surface HER2 expression through trastuzumab-mediated endocytosis and degradation of the HER2 receptor with the result being inhibition of downstream PI3K and MAPK signalling cascade [228]. Other mechanisms that contribute to trastuzumab efficacy include antibody-dependent cellular cytotoxicity, in particular a role for the action of tumour-associated natural killer cells has been described, and suppression of antiapoptotic pathways [230]. Other studies have shown that trastuzumab can increase DNA strand breaks and cause cell cycle perturbations and can cause inhibition of cell growth and arrest in the G1 phase of the cell cycle [226, 231]. We have made use of trastuzumab therapy to target HER2 expressing cells from MCF7 and T47D cell lines and have noted that cell surface expression of HER2 is decreased after treatment without noticeable effects on survival of cells or changes in cell cycle compared to control treated cells for these cell lines. Our treatment with trastuzumab did affect the ability of BT-474 cells to grow and this is to be expected since BT-474 cells express more HER2 and in the clinical setting would be HER2+. Perhaps more surprising was the finding that trastuzumab treatment did not affect sphere formation in BT-474 cells at passage one. Perhaps the cells that are resistant to trastuzumab treatment are sphere forming cells (and potentially cancer stem cells), and these cells might not in fact express as much HER2 as other treatment sensitive cells. Further work will be required to address this issue. Within this thesis, we made use of MCF7 and T47D because they expressed some amount of HER2 without being as affected by its treatment in terms of viability. This is important because it allowed us to test sphere formation following exposure to trastuzumab without viability being a major concern. We found that sphere forming efficiency was not affected by exposure of trastuzumab to these cell lines. This is not exactly what we expected to happen based on the results by Korkaya et al. showing an increase in HER2 being important for
sphere formation. The lines that we used are in many ways trastuzumab resistant - in other words adherent cell lines grown with trastuzumab were found to have similar growth potential as control cells. Korkaya et al. point out that the effects of HER2 overexpression on breast cancer stem cells could be blocked by trastuzumab treatment only in sensitive cell lines and not resistant ones and that this effect is mediated by the PI3-kinase Akt pathway [85]. It is possible that that the use of these two trastuzumab “resistant” cell lines is not optimal. However, when conducting an experiment using BT-474 cells which are sensitive to trastuzumab treatment, we found a non-statistically significant different decrease in sphere formation for trastuzumab treated cells compared to controls. This perhaps does justify the use of MCF7 and T47D cells for this model and shows that HER2 expression is not the only marker that is important in sphere formation. These results are perhaps more in line with what we expected from results by Li et al. showing that the use of Lapatinib, a HER2/EGFR inhibitor, in the neoadjuvant setting resulted in a non-statistically significant decrease in the cancer stem cell compartment (as assessed by a decrease in stem cell self-renewal or sphere formation) while treatment with chemotherapy resulted in a significant increase in sphere formation comparing baseline sphere formation to after treatment sphere formation. Our findings do not rule out the potential importance of HER2 as a marker for BCSCs nor do they rule out the use of trastuzumab for the targeting BCSCs. Importantly, the mechanism of action of trastuzumab may differ between the ex vivo and in vivo situation. More research will need to be conducted to explore the importance of HER2 in BCSCs biology and the use of trastuzumab as a therapy for targeting BCSCs. Chapter Eight of this thesis will further discuss the use of trastuzumab to target HER2 expressing cells.

5.4 Conclusions

These findings validate the use of the tumoursphere assay for characterising stem cell-like cells. We have identified sphere forming capacity in a wide range of breast cancer cell lines representing a variety of molecular subtypes. We have characterised the morphological and phenotypic differences between basal-like and luminal-like spheres, demonstrating that spheres are not homogenous and represent to a great degree the parental cell line phenotype. We have proposed that the culture of tumourspheres is an attractive model to more faithfully represent the in vivo growth of tumour cells compared to monolayer culture and have demonstrated how tumourspheres are similar to structures grown using matrigel. We have shown that the CD44+CD24− phenotype is associated with basal-like cells and not necessarily breast cancer stem cells. Additionally, we have identified the increased expression of MUC1 on luminal spheres,
representing a potential future target for immunotherapy/biotherapy against breast cancer stem cells. We have confirmed that CD49f expression is increased on MCF7 cells grown as spheres compared to adherent cells and have further validated the increased expression of this molecule on other cell lines grown as spheres. We have also assessed the expression of EpCAM on luminal and basal spheres and have shown that luminal spheres express an increase in EpCAM at P2 compared to matched adherent cells and that this does not occur for basal lines. We have finally assessed the expression of HER2 on tumourspheres compared to adherent cells and the potential to target this molecule using trastuzumab treatment. Much work still remains in identifying universal markers of cancer stem cells or breast cancer stem cells. Importantly, the tumorigenicity of cells expressing many of these markers need to be further defined in xenograft assays to definitively label them as cancer stem cell markers. Further characterisation of sphere antigens, and in particular sampling sources from a variety of clinical/molecular subtypes of cancer, will lead to the development of better therapeutics designed to specifically target cancer stem cells that should find application in future clinical trials.
Chapter Six: Use of Proteomics to Identify Putative Breast Cancer Stem Cell Markers

6.1 Introduction

The proteome of a cell, or a group of cells grown under the same conditions, can be defined as the combined set of proteins being expressed by the genomes of those cells at a particular time [232]. Proteomics is the large-scale high-throughput application of proteome research, taking into account protein isoforms and modifications, the structure of proteins, and the interaction between proteins in the analysis (reviewed in [233]). Briefly, proteomics is often accomplished using a two-step approach. In the first step, 2D gel electrophoresis is performed utilising isoelectric focusing and molecular weight separation using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). In the second step, proteins are identified using mass spectrometry. The study of the proteomes of cells can be used to compare two groups of cells to identify differences between them. Applying the technique of proteomics to investigating cancer stem cells will be of use in identifying differences in cell signalling pathways and cell surface phenotype between cancer stem cells and non cancer stem cells. Identification of cell surface phenotypes is particularly important as this can be used to further isolate cancer stem cells for additional research or as a target of therapy. Unfortunately, identification of plasma membrane proteins is often quite difficult to perform due to several reasons: (i) they are often more hydrophobic and therefore less soluble than cytosolic proteins; (ii) they often have significant post-translational modifications (such as glycosylation, phosphorylation and lipid moieties) making identification more difficult; and (iii) they are not as relatively abundant as other proteins (reviewed in [234]). Despite these shortcomings in identifying cell surface markers, proteomics still offers a powerful high-throughput technique to collect a large dataset of proteins to further investigate and test hypotheses with. Utilising proteomics can also compliment other approaches of investigation such as FACS analysis. Proteomic approaches to investigating breast cancer have been performed using both patient samples and cell culture lines, and this has led to the identification of several markers and signalling pathways involved in disease (reviewed in [235]).

Cell lines are not homogenous. Like solid cancers of the body, they represent heterogenous populations of cells. In the field of cancer stem cells it is often ideal to isolate populations based
on cell surface phenotype such as CD49f or HER2 expression, or the expression of a marker such as ALDH1 that is believed to enrich for cancer stem cells over non cancer stem cells (discussed elsewhere in this thesis). However, in the absence of definitive markers for cancer stem cells, it is possible to rely on tumoursphere-promoting culture to enrich for cells with some of the characteristics of cancer stem cells (as has previously been discussed in this thesis). The approach of using whole populations of tumourspheres instead of individual sorted cells is likely to have advantages and disadvantages. One benefit is that by not isolating single populations of cells it might be possible to get a broader view of what the overall changes in tumoursphere culture are compared to traditional adherent culture of cells. Additionally, it is possible that multiple different subpopulations would have the characteristics of cancer stem cells so isolating only one population is too narrow when first searching for relative markers. One disadvantage of this approach is that by using all the cells in a tumoursphere, cells that are the long-term proliferating cells are mixed with short term proliferating cells and differentiated cells. The markers identified in this approach may or may not be enriched within the true cancer stem cell pool. Ultimately, it is critical to show that the markers identified as a putative cancer stem cell markers can isolate cells that have the characteristics of cancer stem cells, both in vitro and in vivo.

Proteome comparison between adherent cells and spheres and between early and late passage spheres was conducted in order to investigate the changes that occur between these populations of cells. We hypothesise that proteins that are upregulated on/within spheres compared to adherent cells might be useful for further isolating subpopulations of cells that might be enriched for the properties of cancer stem cells. Similarly, comparing the proteome of late passage spheres to early passage spheres might identify proteins that are enriched on sphere forming cells, cells that have some of the characteristics of cancer stem cells. MCF7 cells are one of the most studied breast cancer cell lines in use and were one of the first cell lines to be grown under sphere promoting conditions [69]. As such, this cell line was used for comparing the proteome of spheres to adherent cells. This approach has identified several interesting candidate proteins that are expressed within spheres, including proteins involved in metabolic pathways that tumours use; proteins with known stem cell associations; and proteins with known cancer associations. One protein identified, galectin-3, was further characterised on tumourspheres using FACS analysis and blocking with a ligand (N-acetylactosamine (LacNAc)) to investigate the effect of this on sphere formation. This was done to demonstrate the utility of a proteome approach in identifying candidate breast cancer stem cell markers. Galectin-3 was considered a candidate protein of interest because of its higher expression in later passages of spheres compared to earlier spheres,
its known roles in cancer progression, its expression on the plasma membrane and its ability to be blocked with small molecules.

6.2 Results

6.2.1 Results of 2D-difference gel electrophoresis (DIGE) for protein expression changes between adherent cells and P6 MCF7 tumourspheres and between P2 MCF7 tumourspheres and P8. Proteins were visually assessed for increased/decreased expression on the gel. Only the most visibly upregulated/downregulated proteins were then identified. Uniprot was used as a guide for protein function/association: http://www.uniprot.org/. Table 6.1 shows proteins upregulated on P6 spheres compared to adherent cells, and P8 spheres compared to P2 spheres. Table 2 shows proteins downregulated on P6 spheres compared to adherent cells, and P8 spheres compared to P2 spheres. A variety of proteins related to metabolism, stress response, regulation of cytoskeleton, stem cell association or breast cancer association were found to be differentially expressed between the populations of cells studied. Figure 6.1 shows a representative 2D-DIGE for P2 spheres compared to P8 spheres, with the increase seen for galectin-3 in P8 spheres over P2 spheres highlighted.

6.2.2 Quantitative comparison of protein expression between adherent cells, passage 2 and passage 5 spheres for three proteins. As a follow-up experiment, the proteome of adherent cells and P2 and P5 spheres were assessed with three biological replicates. Three proteins were quantitatively assessed for protein expression level by spectral counting. Mass spectrometry-driven proteomics has been used for a variety of studies [236, 237] and spectral counting provides a quantitative measurement for comparing protein abundance between samples [238, 239]. Comparison of spectral counts amongst these samples indicated that pyruvate kinase isozymes M1/M2, fructose 1,6 bisphosphotase, and galectin-3 were increased within late passage spheres compared to adherent cells (Figure 6.2), complimenting previously obtained qualitative results for different samples.
### Table 6.1

**Proteins with increased expression on P6 spheres compared to adherent cells**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function/Association</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate kinase isozymes M1/M2</td>
<td>Metabolism</td>
</tr>
<tr>
<td>Fructose-1,6 bisphosphatase</td>
<td>Metabolism</td>
</tr>
<tr>
<td>Glucose-6-phosphate 1-dehydrogenase (G6PD)</td>
<td>Metabolism</td>
</tr>
<tr>
<td>Methylcrotonoyl-CoA carboxylase beta chain, mitochondrial</td>
<td>Metabolism</td>
</tr>
<tr>
<td>Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial</td>
<td>Metabolism</td>
</tr>
<tr>
<td>6-phosphogluconolactonase</td>
<td>Metabolism</td>
</tr>
<tr>
<td>Peptidyl-prolyl cis-trans isomerase A (EC 5.2.1.8)</td>
<td>Metabolism</td>
</tr>
<tr>
<td>Catechol O-methyltransferase</td>
<td>Inactivator of some neurotransmitters and hormones</td>
</tr>
<tr>
<td>Peptidyl-prolyl cis-trans isomerase B</td>
<td>Protein folding</td>
</tr>
<tr>
<td>Endoplasmic reticulum resident protein 29</td>
<td>Protein folding</td>
</tr>
<tr>
<td>Hypoxia up-regulated protein 1</td>
<td>Stress response</td>
</tr>
<tr>
<td>Protein disulfide-isomerase</td>
<td>Stress response</td>
</tr>
<tr>
<td>Heat-shock protein beta-1 (Hsp27) (Heat shock 27 kDa protein)</td>
<td>Stress response</td>
</tr>
<tr>
<td>Proteasome subunit beta type-4</td>
<td>Stress response, protein degradation</td>
</tr>
<tr>
<td>Ezrin</td>
<td>Breast cancer association</td>
</tr>
<tr>
<td>Latexin</td>
<td>Stem cell association</td>
</tr>
<tr>
<td>Gelsolin/Brevin</td>
<td>Regulation of actin, cytoskeleton</td>
</tr>
</tbody>
</table>

**Proteins with increased expression on P8 spheres compared to P2 spheres**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function/Association</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fructose-1,6-bisphosphatase</td>
<td>Metabolism</td>
</tr>
<tr>
<td>Aldo-keto reductase family 1 member C2</td>
<td>Metabolism</td>
</tr>
<tr>
<td>2,4-dienoyl-CoA reductase, mitochondrial</td>
<td>Metabolism</td>
</tr>
<tr>
<td>Sialic acid synthase</td>
<td>Metabolism</td>
</tr>
<tr>
<td>Very long-chain specific acyl-CoA dehydrogenase, mitochondrial</td>
<td>Metabolism</td>
</tr>
<tr>
<td>Bifunctional 3’-phosphoadenosine 5’-phosphosulfate synthase 2</td>
<td>Metabolism</td>
</tr>
<tr>
<td>Isocitrate dehydrogenase [NADP] cytoplasmic</td>
<td>Metabolism</td>
</tr>
<tr>
<td>Proteasome activator complex subunit 2</td>
<td>Stress response</td>
</tr>
<tr>
<td>Proteasome activator complex subunit 1</td>
<td>Stress response</td>
</tr>
<tr>
<td>Annexin A5 (ANXA5)</td>
<td>Stress response, feature of apoptosis</td>
</tr>
<tr>
<td>26S protease regulatory subunit 7 (PR57)</td>
<td>Stress response, protein degradation</td>
</tr>
<tr>
<td>Heat shock-related 70 kDa protein 2 (Hsp70)</td>
<td>Stress Response</td>
</tr>
<tr>
<td>Ubiquitin-like modifier-activating enzyme 1</td>
<td>Stress Response</td>
</tr>
<tr>
<td>Interferon-induced 17 kDa protein</td>
<td>Ubiquitin-like protein, cell-cell signalling</td>
</tr>
<tr>
<td>Chloride intracellular channel protein 1</td>
<td>Forms chloride channels in cells</td>
</tr>
<tr>
<td>Endophilin B2 (SHLB2)</td>
<td>-</td>
</tr>
<tr>
<td>SH3 domain-binding glutamic acid-rich-like protein</td>
<td>-</td>
</tr>
<tr>
<td>Alcohol dehydrogenase class-3</td>
<td>-</td>
</tr>
<tr>
<td>Alcohol dehydrogenase [NADP+] (AK1A1)</td>
<td>-</td>
</tr>
<tr>
<td>Selenium-binding protein 1</td>
<td>Protein transport</td>
</tr>
<tr>
<td>Endoplasmic reticulum resident protein 29</td>
<td>Protein folding, secretion from ER</td>
</tr>
<tr>
<td>Keratin, type 1 cytoskeletal 13</td>
<td>Keratin expressed in luminal cells of mammary gland ducts</td>
</tr>
<tr>
<td>Keratin type 1 cytoskeletal 10</td>
<td>Keratin</td>
</tr>
<tr>
<td>Keratin type 1 cytoskeletal 20</td>
<td>Keratin</td>
</tr>
<tr>
<td>Tubulin alpha-1C/B/A</td>
<td>Components of microtubules, cytoskeleton</td>
</tr>
<tr>
<td>Macrophage-capping protein</td>
<td>Blocks actin filaments, cytoskeleton</td>
</tr>
<tr>
<td>Gelsolin/Brevin</td>
<td>Regulation of actin, cytoskeleton</td>
</tr>
<tr>
<td>Galectin-3</td>
<td>Breast cancer association</td>
</tr>
<tr>
<td>Serpin B5</td>
<td>Tumour suppressor breast cancer association</td>
</tr>
<tr>
<td>Ezrin-radixin-moesin-binding phosphoprotein 50</td>
<td>Scaffold protein, breast cancer association</td>
</tr>
</tbody>
</table>

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Table 6.1. Proteins upregulated on MCF7 P6 spheres vs. adherent cells or P8 vs. P2 spheres.
Table 6.2

<table>
<thead>
<tr>
<th>Proteins with decreased expression on P6 spheres compared to adherent cells</th>
<th>Function/Association</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphoglycerate mutase 1</td>
<td>Metabolism</td>
</tr>
<tr>
<td>Triosephosphate isomerase</td>
<td>Metabolism</td>
</tr>
<tr>
<td>Probable isocitrate dehydrogenase [NAD] subunit beta</td>
<td>Metabolism</td>
</tr>
<tr>
<td>Adenylate kinase isoenzyme 4, mitochondrial</td>
<td>Kinase</td>
</tr>
<tr>
<td>Eukaryotic translation initiation factor 5A-2</td>
<td>Protein biosynthesis</td>
</tr>
<tr>
<td>Heterogeneous nuclear ribonucleoproteins A2/B1 mRNA processing</td>
<td>mRNA processing</td>
</tr>
<tr>
<td>Peptidyl-prolyl cis-trans isomerase FKBP4</td>
<td>Component of steroid receptor complexes</td>
</tr>
<tr>
<td>ATP-dependent DNA helicase 2 subunit 2</td>
<td>DNA repair, chromosome translocation</td>
</tr>
<tr>
<td>Peptidyl-prolyl cis-trans isomerase B</td>
<td>Protein folding</td>
</tr>
<tr>
<td>78 kDa glucose-regulated protein</td>
<td>Protein assembly</td>
</tr>
<tr>
<td>Flotillin-1</td>
<td>Scaffolding protein</td>
</tr>
<tr>
<td>TAR DNA-binding protein 43</td>
<td>Regulation of transcription and splicing</td>
</tr>
<tr>
<td>Ran-specific GTPase-activating protein</td>
<td>intracellular signalling, cell cycle progression</td>
</tr>
<tr>
<td>GTP-binding nuclear protein Ran</td>
<td>Cell cycle, mitosis, protein transport</td>
</tr>
<tr>
<td>Proteasome subunit beta type-8</td>
<td>Stress response, protein degradation</td>
</tr>
<tr>
<td>Antigen peptide transporter 2</td>
<td>Immune function, antigen transport</td>
</tr>
<tr>
<td>BAG family molecular chaperone regulator 1</td>
<td>Inhibits Hsp70, anti-apoptotic function</td>
</tr>
<tr>
<td>Nucleophosmin</td>
<td>Protein chaperoning, cell proliferation</td>
</tr>
<tr>
<td>Gluthathione transferase omega-1</td>
<td>-</td>
</tr>
<tr>
<td>S-formylglutathione hydrolase</td>
<td>-</td>
</tr>
<tr>
<td>14-3-3 protein epsilon isoform transcript variant 1</td>
<td>-</td>
</tr>
<tr>
<td>Tubulin beta-6</td>
<td>Component of microtubules, cytoskeleton</td>
</tr>
<tr>
<td>Tropomyosin alpha-4 chain</td>
<td>Stabilisation of actin filaments in the cytoskeleton</td>
</tr>
<tr>
<td>Tropomyosin alpha-3 chain</td>
<td>Stabilisation of actin filaments in the cytoskeleton</td>
</tr>
<tr>
<td>LIM and SH3 domain protein 1</td>
<td>Regulation of actin, cytoskeleton</td>
</tr>
<tr>
<td>Cofilin-1</td>
<td>Actin polymerization and depolymerization, cytoskeleton</td>
</tr>
<tr>
<td>PRKC apoptosis WTI regulator protein</td>
<td>Pro-apoptotic function</td>
</tr>
<tr>
<td>Protein SET</td>
<td>Anti-apoptosis</td>
</tr>
<tr>
<td>Stathmin</td>
<td>Regulation of microtubules, cell differentiation, cell cycle, breast cancer association</td>
</tr>
<tr>
<td>Anterior gradient protein 2 homolog</td>
<td>Breast cancer association</td>
</tr>
<tr>
<td>Transgelin-2</td>
<td>Tumour suppressor role in breast cancer</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Proteins with decreased expression on P8 spheres compared to P2 spheres</th>
<th>Function/Association</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl-CoA acetyltransferase, cytosolic</td>
<td>Metabolism</td>
</tr>
<tr>
<td>Cystathionine beta-synthase</td>
<td>Metabolism</td>
</tr>
<tr>
<td>Cathepsin D</td>
<td>Hydrolase/protease</td>
</tr>
<tr>
<td>Spermidine synthase</td>
<td>Transferase</td>
</tr>
<tr>
<td>Hypoxia up-regulated protein 1</td>
<td>Stress Response</td>
</tr>
<tr>
<td>26S protease regulatory subunit 7</td>
<td>Stress response, protein degradation</td>
</tr>
</tbody>
</table>

Table 6.2. Proteins downregulated on MCF7 P6 spheres vs. adherent cells or P8 vs. P2 spheres.
Figure 6.1

MCF7 spheres Passage 2 (green) vs. Passage 8 (red)

Figure 6.1. 2D DIGE for P2 vs. P8 MCF7 spheres demonstrating an increased expression of Galectin-3 for P8 spheres. DIGE (Differential Gel Electrophoresis) results showing differential protein expression between P2 and P8 spheres from MCF7 cells. Green indicates increased expression for P2, red indicates increased expression for P8, and yellow indicates no change in expression. Red circle indicates protein expression for Galectin-3, demonstrating an increase between P2 and P8. Gel done by Marcus Hastie, Protein Discovery Centre, QIMR.
Figure 6.2. Spectral Counts of Adherent Cells versus Passage 2 and Passage 5 Spheres for three proteins of interest. Pyruvate Kinase Isozymes M1/M2 increased expression on P5 spheres compared to adherent cells \( p = 0.0031, ** \). Fructose-1,6 bisphosphatase increased expression on P5 spheres compared to adherent cells \( p<0.0001, *** \). Galectin-3 increased expression on P5 spheres compared to adherent cells \( p = 0.0039, ** \).
6.2.3 **The effect of LacNAc on sphere culture.** P2 spheres were grown for 5 days in the presence of 0, 0.1, 0.3, 1, or 3 mM LacNAc (a ligand with potential utility as an inhibitor of galectin-3), and morphology of cells/spheres and sphere forming efficiency was assessed. MCF7 spheres cultured with LacNAc demonstrated an increase in cells adhering to the culture flask and a decrease in cells forming spheres. This decrease was dose dependent. MDA-MB-436 cells cultured in sphere-promoting conditions did not demonstrate these changes when co-cultured with LacNAc (Figure 6.3). MCF7 cells demonstrated a statistically significant decrease in sphere formation with the addition of LacNAc (p ≤ 0.0001) (Figure 6.4a). MDA-MB-436 cells sphere forming efficiency was not altered by the addition of LacNAc (Figure 6.4a). In a different study screening more cell lines and using a higher concentration of LacNAc (0, 1, 3, 6 mM); MCF7 cells and MDA-MB-436 cells sphere forming efficiency were confirmed to be responsive and non-responsive respectively to the addition of LacNAc (Figure 6.4b). Additionally, co-culture of HBL-100 cells with LacNAc was found to decrease sphere forming efficiency, while no effect was seen for BT-474 cells or SUM-159-PT cells (Figure 6.4b).

6.2.4 **Galectin-3 expression on P2 tumourspheres.** P2 day 5 MCF7 and MDA-MB-436 spheres were separated into single cells and analysed for viability and expression of galectin-3 compared to matched adherent cultures (Figure 6.5a). Cells from adherent culture were found to be more viable than single cells from tumoursphere-promoting conditions for both MCF7 and MDA-MB-436 (p ≤ 0.0008). MCF7 spheres were also found to be more viable than MDA-MB-436 cells (p ≤ 0.0001). Cell surface expression of galectin-3 on viable cells was found to be low for both adherent cells and cells grown in tumoursphere-promoting conditions for both cell lines (Figure 6.5b). However, galectin-3 expression was found to be high for cells that stained as dead/dying using PI. This could be due to galectin-3 being increased on the surface of these cells or staining in nuclear membrane. MCF7 dead/dying cells were found to exhibit more staining for galectin-3 then MDA-MB-436 cells.
Figure 6.3. LacNAc effect on phenotype of MCF7 and MDA-MB-436 tumourspheres. Representative 4x photomicrographs of P2 spheres five days after culture in LacNAc containing NSA media, as previously described (experiment done two times with similar result). MCF7 spheres cultured with LacNAc show a more differentiated morphology than those not cultured in LacNAc with most cells no longer forming spheres and attaching to the culture flask as seen under adherent conditions. (a) No Tx, (b) 0.1 mM, (c) 0.3 mM, (d) 1 mM, and (e) 3mM LacNAc. No such trend was seen for MDA-MB-436 cells. (f) No Tx, (g) 0.1 mM, (h) 0.3 mM, (i) 1 mM, and (j) 3mM LacNAc. Bars represent 100 μm.
Figure 6.4

(a) LacNAc demonstrated an ability to prevent sphere formation for MCF7 cells in a dose dependent manner. A one-way ANOVA with a post-test for linear trend between number of spheres generated and amount of LacNac added demonstrated a significant trend, *** p ≤ 0.0001. LacNAc was not found to have a significant effect on MDA-MB-436 cells. Experiment repeated twice, representative results shown. (b) Results utilising a larger amount of LacNac demonstrated a similar trend for MCF7 and MDA-MB-436 cells. HBL-100 cells were also found to have decreased with exposure to LacNac while SUM-159-PT and BT-474 cells were not. Experiment completed one time. Bars represent SEM.
Figure 6.5. Viability of MCF7 and MDA-MB-436 cells from tumourspheres and adherent cells and expression of galectin-3. Passage 2 day 5 spheres and matched adherent cells were analysed for viability using PI and expression of galectin-3. (a) Spheres were determined to be less viable than adherent cells for both cell lines (p ≤ 0.0008, ***). MCF7 spheres were determined to be more viable than MDA-MB-436 spheres (***), and adherent cells were determined to have similar levels of viability. Bars represent 100 μm. (b) Galectin-3 expression was determined to be mainly expressed by dead/dying cells. Extracellular expression of galectin-3 by viable cells at P2 was not found to be increased over expression of adherent cells and MCF7 single cells from tumourspheres expressed similar amounts of galectin-3 as MDA-MB-436 cells. MCF7 cells stained as dead/dying expressed more galectin-3 than MDA-MB-436 dead/dying cells.
6.2.5 Effect of LacNAc on P6 MCF7 tumoursphere viability, galectin-3 expression and cell cycle phenotype. P6 tumourspheres were cultured with LacNAc over a dilution of concentrations and viability, and galectin-3 expression was assessed at 48 hours and 5 days. P6 tumourspheres were chosen as galectin-3 expression was noted both by DIGE comparison and FACS to increase in expression over serial passage. For these experiments, a “liberal” gating strategy was employed to assess the viability of cells and subsequently the expression of galectin-3. This gate included some cells that stained slightly over background for expression of PI. This was done because (i) cells at 48 hours mostly demonstrated some PI staining above background (possibly due to the nature of NSA culture in which 90% of cells are thought to die during passage leaving only growth factor-responsive cells to self-renew); and (ii) there was a clear discrimination between cells with slight PI staining (the cells included in some of the subsequent analysis) and cells with much higher PI staining (truly dead cells). A “conservative” gating strategy analysing only clearly live cells was also employed for some of the analysis (Figure 6.7a for examples of gating strategies). At 48 hours using a liberal gating strategy there was no effect of LacNAc upon cell viability, positivity of galectin-3 expression over background isotype staining, or mean fluorescent intensity (MFI) (Figure 6.6a). At 5 days using a liberal gating strategy there was no effect of LacNAc upon viability of cells (Figure 6.6b). There was; however, an effect of addition of LacNAc upon positivity of galectin-3 expression and MFI with a decreasing trend in expression upon addition of LacNAc ($p \leq 0.0001$). Using a more conservative gating strategy upon the same dataset demonstrated an increase in viability of cells upon the addition of LacNAc (Figure 6.6c). A similar decrease in galectin-3 expression was noted by both positivity and MFI upon the addition of LacNAc ($p \leq 0.0001$).
**Figure 6.6. LacNAc effect on viability and galectin-3 expression for P6 MCF7 tumourspheres.** MCF7 spheres were cultured in LacNAc at various concentrations for 48 hours or 5 days and then analysed for viability by PI, expression of live-gated cells cell surface galectin-3 positivity above isotype background staining and mean fluorescent intensity (MFI). At 48 hours (a) there was no effect of LacNAc on any of these parameters when using a liberal gate to look at viability (i.e. some cells stained above background for PI were included in analysis). At 5 days (b) using a liberal gate for viability there was no effect on viability by the addition of LacNAc up to 1 mM. A one-way ANOVA with a post-test for linear trend between galectin-3 MFI or positivity and amount of LacNAc added demonstrated a significant trend of decreasing expression of galectin-3 upon co-culture with LacNAc (***, p ≤ 0.0001). At 5 days (c) and using a more conservative gate for viability, there was an increase in viability of cells upon usage of LacNAc, and a decrease in galectin-3 expression by positivity (**, p = 0.0029) and MFI (***). Bars represent SEM.
Figure 6.7. Gating strategy for LacNAc experiment and cell cycle analysis after LacNAc treatment for P6 MCF7 tumourspheres. MCF7 spheres were cultured in LacNAc at various concentrations for 48 hours or 5 days and then analysed for cell cycle. (a) Gating strategy for analysing galectin-3 and viability after LacNAc culture. Cells were sorted for forward (FSC) and side (SSC) scatter characteristics, then for viability before analysing galectin-3 expression. Preparing single cells from sphere culture for analysis often times results in an increase in dead cells. In some cases, cells with staining above background for viability were analysed. (b) Cell cycle plots show percentage of cells in G_1 (full), S (empty) and G_2 (hatched) phases (n = 3 repeats). No significant differences were noted at either 48 hours or 5 days after the start of culture for treatment with LacNAc. Bars represent SEM.
6.3 Discussion

6.3.1 Proteins upregulated on spheres compared to adherent cells. In order to investigate the proteome of tumourspheres compared to adherent cells, we made use of MCF7 cells grown under both conditions and compared the proteome using 2D-DIGE. P6 tumourspheres were chosen to be compared to adherent cells because later passage of spheres might allow for enrichment of sphere forming cells. Results investigating proteins upregulated in spheres revealed several candidate proteins of interest related to metabolic functions of the cell, stress response, stem cell or breast cancer association, or cytoskeletal changes. Results for proteins downregulated on spheres compared to adherent cells are briefly presented here. This work is exploratory in nature. Current research is ongoing to confirm these proteome results.

6.3.1.a Proteins involved in metabolism. Several proteins involved in metabolic functions were found to have increased expression within tumourspheres compared to adherent cells. In general these increases could be attributed to increased metabolism or different metabolic requirements for spheres compared to adherent cells. Pyruvate kinase isozymes M1/M2 were found to be increased within spheres compared to adherent cells. Pyruvate kinase isozymes M1/M2 have a previously described role in tumour formation with a shift from M1-PK to M2-PK expression occurring within tumour cells [240-242]. M2-PK occurs in a high affinity highly active tetrameric form found in normal proliferating cells and a low affinity nearly inactive dimeric form present in tumour cells. The quantification of tumour M2-PK in plasma [243, 244] and stool [245] has been used as a tool for early detection of a variety of tumours and follow-up of tumours post therapy. Indeed, tumour M2-PK determination in the plasma of patients with metastasised breast cancer has been found to be a useful tool for monitoring therapeutic success following trastuzumab therapy [246]. In the active form, pyruvate kinase binds its substrate phosphoenolpyruvate and aids the conversion of glucose to pyruvate under the production of energy (glycolysis). Highly proliferating cells exploit the shift to the inactive form of M2-PK as the glycolytic intermediates above pyruvate kinase accumulate and are used instead to form amino acids, phospholipids, and nucleic acids, all requirements of highly proliferating cells [247, 248] (Figure 6.8). Thus pyruvate kinase, and in particular M2-PK, is situated at a key spot in glycolysis, as the dimer/tetramer ratio of M2-PK helps determine whether glucose carbons are converted to pyruvate and lactate under the production of energy or utilised in synthetic
processes. Results of the DIGE indicate that the M2 isoform has likely been identified. The results do not; however, indicate if M2-PK is present as dimers or tetramers. It might be reasonable to infer that M2-PK in spheres is present in the inactive dimer.

Figure 6.8


**Figure 6.8. The consequences of Tumour M2-PK.** Pyruvate kinase isozymes M1/M2 and fructose-1,6 bisphosphatase were found to be increased within spheres compared to adherent cells. These changes are associated with an increase in proliferation within tumour cells. In normal proliferating cells in the active form, pyruvate kinase (as M2-PK tetramer or M2-PK) binds its substrate phosphoenolpyruvate and aids the conversion of glucose to pyruvate under the production of energy (glycolysis). In highly proliferating or tumour cells there is a shift to the inactive form of M2-PK (dimer) and the glycolytic intermediates above pyruvate kinase accumulate (including fructose-1,6 bisphosphatase, blue circle) and are used instead to form amino acids, phospholipids, and nucleic acids, all requirements of highly proliferating cells.
form as the expression of a phosphometabolite, fructose-1,6 bisphosphotase, upstream of pyruvate kinase was also found to be increased. Indeed, high levels of fructose-1,6 bisphosphotase is part of regulatory mechanism of the metabolic budget system that will induce the dimer form of M2-PK to switch to the tetramer form once some critical level is reached that requires the cell to produce energy [247-249]. Taken together, these results suggest that spheres have an increase in the tumour form of M2-PK compared to adherent cells. This would then indicate that spheres are enriched for cells that have a proliferative requirement compared to adherent cells. This could in turn reflect the nature of the tumoursphere growth as being driven by cancer stem cells, which produce dividing daughter transit-amplifying cells and which have an extensive proliferative potential.

6.3.1.b Proteins involved in stress response. Another protein found increased within spheres compared to adherent cells was heat shock protein beta-1 (Hsp27). Heat shock proteins are ubiquitous molecules that are often upregulated in response to sublethal heat or other stresses and are involved in protecting cells from stress by binding to partially denatured proteins, dissociating protein aggregates, or regulating proper protein folding and transport of newly synthesised proteins (reviewed in [250, 251]). Heat shock proteins also have a described role in cell proliferation and differentiation as well as in disease prognosis and drug resistance in breast cancer. High levels of Hsp27 have been found in breast cancer patient’s sera [252], and levels of Hsp27 have been found to be higher in tumour cells than in nontransformed cells from a variety of cancers including breast, ovarian, and leukaemias [253]. Hsp27 has been shown to be involved in drug resistance in breast cancer patients that have been treated with combination chemotherapy, possibly by virtue of their chaperone function by “guarding” nascent polypeptides and reversing/decreasing degraded protein states [254]. High expression of Hsp27 has been noted to correlate with metastasis, poor prognosis and resistance to chemotherapy and radiation in breast and endometrial cancer [254, 255], all of which are also hallmarks of tumour-initiating cancer stem cells. In breast cancer Hsp27 expression has been associated with an increase in invasiveness but a decrease in cell motility [256], and overexpression has sometimes been correlated with a low proliferation index in patient samples [257]. Overexpressed Hsp27 prevents cell death that has been triggered by a variety of stimuli including chemotherapy/cytotoxic drugs. One way that this occurs is through an anti-apoptotic function. Hsp27 can function as an inhibitor of caspase activation, in particular caspase-3 [258, 259]. The mechanism for this role involves the ability of Hsp27 to prevent the formation of the apoptosome and concurrent activation of caspases [260]. Another way Hsp27 prevents apoptosis is by favouring the degradation of certain proteins.
under stress conditions. For instance, Hsp27 under stress conditions favours the ubiquitination and degradation of IκBα, an NFκB inhibitor. As a consequence, there is a subsequent increase in the amount of NFκB activity leading towards prosurvival signalling [261]. Another mechanism in place under stressful conditions is to favour the ubiquitination of p27kip1. The consequence of the degradation of that molecule is that cells do not accumulate in the G0/G1 phase of the cell cycle but rather in the S phase, and are therefore more ready to rapidly restart proliferation once stress conditions have been removed [262]. The chaperone function of Hsp27 is not the only way that it aids preventing apoptosis. One function of Hsp27 is in enhancing the catalytic activity of the 26S proteasome machinery in order to increase the degradation of irreversibly denatured proteins, which have been ubiquitinated, following cellular stress. The proteasomal degradation pathway is necessary for many of the functions of cells including regulation of cell cycle, gene expression, and response to oxidative stress. The 26S proteasome is composed of a catalytic 20S subunit and a regulatory 19S subunit. Hsp27 has been shown to interact directly with the 19S subunit [261]. Interestingly, we found an increase in a constituent of the 20S subunit, the proteosome subunit β type 4, giving further credence to the concept that Hsp27 is upregulated in the spheres, perhaps as a result of the stress of tumoursphere-promoting conditions and as a potential anti-apoptotic mechanism by regulating protein degradation.

Protein disulphide-isomerase (PDI) was also found to be upregulated within spheres compared to adherent cells. PDI functions to catalyse protein folding by catalysing the formation and breakage of disulphide bonds between cysteine residues within proteins [263]. PDI acts as chaperone protein to aid wrongly-folded proteins; as such it might also be an integral part of the cellular machinery dealing with misfolded proteins following stress. Furthermore, 150 kDa oxygen-regulated protein precursor (Orp150) (Hypoxia upregulated 1) was also found to be increased in tumourspheres. This molecule is upregulated under hypoxic conditions or stressful conditions. It is not likely that hypoxic conditions would affect spheres significantly greater than adherent cells, and as other proteins such as hypoxia-inducible factor (HIF)-1α were not found to be upregulated, it is most likely the case that the upregulation of this molecule is a result of stressful conditions of sphere culture (hypoxia reviewed in [264]). Taken together these results demonstrate that spheres have increased anti-apoptotic machinery in place compared to adherent cells. This could be due to the stressful nature of the tumoursphere-promoting conditions, in particular the lack of serum supplementation compared to adherent conditions. However, it is also possible that these conditions are more faithfully modelling the in vivo situation of tumours and, as such, changes seen in tumoursphere-promoting conditions are important for investigation. Regardless, these
findings are intriguing in light of the fact that Hsp27 is a potential pharmacological target for cancer therapy and the finding that Hsp27 might be upregulated on cell populations enriched for cancer stem cells.

6.3.1.c Proteins with stem cell function. Another molecule found upregulated within spheres compared to adherent cells was latexin. Latexin is the only identified endogenous carboxypeptidase A inhibitor in mammals and has recently been identified to influence the size of the hematopoietic stem cell (HSC) pool [265]. Latexin has been shown to be a negative regulator of the stem cell pool acting through two mechanisms: (i) it decreases HSC cell replication; and (ii) it increases HSC apoptosis (reviewed in [266]). Thus, at least in the hematopoietic system and perhaps in other systems, latexin is critically involved in the regulation of stem cells. Mechanisms by which latexin is involved in stem cell regulation include such things as: (i) inhibition of carboxypeptidase A; (ii) regulating intracellular signalling pathways; and (iii) regulating protein folding and aggregation. Young stem cells do not express as much latexin as older stem cells, and stem cell functions are “normal” resulting in a situation of homeostasis of self-renewal. As stem cells age latexin levels increase and there is a shift in the balance between proliferation and apoptosis towards apoptosis. Latexin-associated biological functions are also affected leading to stem cell senescence, loss, or inappropriate regulation. Increased levels of latexin in aged stem cells may also play a role in cancer formation, especially in light of the fact that older stem cells might be more likely to incur mutagenic events than younger stem cells. Latexin is a member of the cystatin superfamily. One member of that family of molecules is cystatin C. Cystatin C has been shown to be influential in the metastatic capacity of melanoma cells in a mouse model [267]. Coincidently, cystatin C has also been shown to regulate neurosphere generation, differentiating of embryonic stem cells into neural stem cells, and as such further lends credence to the idea of latexin being involved in sphere formation and stem cell regulation [268]. It has been put forth by others that latexin, as a member of the same superfamily of molecules as cystatin C, might play a similar role in the malignant transformation of stem/progenitor cells into tumour-initiating/cancer stem cells [266]. It has been hypothesised that this occult role that latexin plays in early tumourigenesis might be the result of epigenetic mechanisms leading to differential expression of latexin [265]. Conversely, latexin has been described as a tumour suppressor protein, as molecules that share sequence similarity with latexin have been shown to be downregulated or absent in a variety of tumour types [266, 269]. Presently, many of the functions of latexin are not fully understood. It is likely that each of the different pathways that latexin is involved in, either independently or synergistically, are
contributing to the regulation of proliferation; stem cell renewal and apoptosis; and potentially to these functions in cancer stem cells. As such, this molecule warrants further investigation in regards to the expression in breast cancer stem cells, perhaps with particular regard to breast cancer stem cells, as they undergo multiple rounds of self-renewal. In our study we have found latexin increased within tumourspheres, cells that have a demonstrated self-renewal capacity. These cells were cultured for 6 passages (roughly 35 days) from the parental adherent cell line. During this time it is possible that the population of self-renewing cells aged to such an extent as to increase expression of latexin. Further research will be needed to elucidate the role(s) that latexin plays in influencing stem cell and cancer stem cell regulation.

**6.3.1.d Proteins with a cytoskeleton function.** The molecule ezrin, a membrane-cytoskeletal crosslinker protein, was also found to be increased on spheres compared to adherent cells. Cancer stem cells are thought to be the cells that are responsible for tumour metastatic disease. Key steps in the spread of tumour metastases include the collapse of cell-cell contact, increased cell motility, and invasion [270]. Ezrin is a member of the ezrin-radixin-moesin family, and as such is involved in cytoskeletal functions such as cell adhesion, survival and motility [271-273]. Members of this family link the actin cytoskeleton to membrane-associated proteins including adhesion molecules such as ICAMs and CD44, amongst others [274]. CD44 has previously been identified as a potential marker of tumour-initiating cells (references elsewhere in this thesis). Ezrin demonstrates strong expression in invasive human cancers including osteosarcomas, melanomas, astrocytic tumours, and pancreatic, lung and endometrial carcinomas [275-278]. Furthermore, studies that have looked at blocking ezrin function have demonstrated that this will abrogate metastatic disease [279, 280]. In relation to breast cancer, ezrin has been shown to be required for metastatic disease in a mouse model of murine mammary carcinoma [281]. In human breast cancer cell lines, ezrin levels have been shown to be similar among various lines [282]. However, apical membrane localisation was noted for low invasive cell lines such as MCF7, whereas ezrin localisation was found to be more diffuse with cytoplasm expression and expression in motile structures (such as filopodia and membrane ruffles) for more metastatic cell lines such as MDA-MB-435S. This study also noted that cytoplasmic ezrin localisation was associated with higher levels of Ki-67 expression, lymph-node metastases, and dedifferentiation leading to invasiveness. It would be interesting to investigate the expression of ezrin via immunohistochemistry in tumourspheres compared to adherent cells to determine if the pattern of ezrin localization is affected by culture conditions. If results indicate that tumourspheres show an increase in ezrin and cytoplasmic ezrin staining compared to adherent
cells (which have previously been shown in the case of MCF7 to stain in the apical membrane), this might indicate a more metastatic phenotype for cells grown under tumoursphere-promoting conditions compared to adherent cells. Indeed, the current result might indicate that this is the case, as membrane proteins were often lost in our protein preparations, thus indicating that spheres most likely express ezrin in the cytoplasm while adherent cells do not. Ezrin activation may be a key prognostic marker for metastatic disease and a potential target for treatment of breast cancer that might target cancer stem cells, those cells that are also metastatic.

An isoform of gelsolin was found to be increased on spheres compared to adherent cells. Gelsolin comes in two isoforms, one that is found in the cytoplasm and cytoskeleton and named gelsolin, and another that is mainly secreted named brevin. Gelsolin is a calcium-dependent actin-binding protein, that plays a role in cell motility, through regulation of actin filament lengths both positively and negatively [283]. As such, gelsolin has been implicated in cytoskeletal changes during differentiation and carcinogenesis. A role for gelsolin in anti-apoptosis has also been described [284]. Brevin is a calcium-dependent protein that severs F-actin filaments into shorter ones and is produced by the same gene that produces gelsolin [285]. Brevin is thought to be mostly secreted; however, brevin might be found in the cytoplasm of cells as well [286]. Secreted brevin is thought to be important in clearing actin from circulation following cell injury or death [287, 288]. Our technique for preparing tumourspheres for protein preparation involved collecting intact spheres. It has been reported elsewhere in this manuscript that lumens form in MCF7 spheres. It is possible that the isoform of gelsolin identified using our approach has identified brevin located in the lumens formed within intact spheres. Protein preparation from adherent cells would not likely find secreted proteins, as no lumens have formed allowing for their sequestering. More careful experimentation would be needed to definitively show that lumens formed within spheres are capable of sequestering brevin. However, if this is the case then brevin might be acting in its role to clear actin. It is also possible that our approach has identified brevin or gelsolin located in the cytoplasm, in which case gelsolin/brevin might be involved in regulating the cytoskeleton in ways important for cell motility or potentially metastasis [286]. Reduced expression of gelsolin has been identified in transformed fibroblasts [289], breast carcinomas [290-292], and non-small cell lung carcinomas [293]. For breast cancer, downregulation of gelsolin has been shown to inversely correlate with the progression of atypical ductal hyperplasia to ductal carcinoma in situ [283]. Therefore, current evidence points to the loss of gelsolin expression as tumours turn from relatively benign phenotype through states of tumourigenesis, likely resulting in disregulation of cell motility, growth, shape and adhesion. If we have identified
cytoplasmic gelsolin, our results would demonstrate that gelsolin has increased expression within spheres, the cells that are enriched for cancer stem cells, and therefore a more metastatic phenotype. This conclusion would not be supported by the reviewed literature. However, it is possible that in the case of sphere culture, gelsolin is a requirement to maintain the cell-cell adhesion that is a hallmark of sphere formation. This makes sense as gelsolin is required for regulating the cytoskeleton of cells which is involved with cellular adhesion. Future experiments are warranted to definitively answer whether gelsolin or brevin was identified in our experiment. It would be interesting in the future to investigate the effects of blockers of gelsolin on sphere morphology or to compare the expression of gelsolin in different subtypes of cancer as spheres from luminal lines and basal lines have a different morphology.

6.3.1.e Proteins downregulated within spheres compared to adherent cells. Several proteins were found to be downregulated within spheres compared to adherent cells. Several of these were related to components of microtubules or the cytoskeleton. These changes might be due to differences in culture technique of growing cells adherent vs. 3D growth as non-adherent spheres. Alternatively, these differences could be due to differences in the cell cycle or motility or metastatic phenotype of the cells grown under each condition. Two proteins that have a role in promoting breast cancer metastases were found to be downregulated in spheres compared to adherent cells. One of these is stathmin, a cell cycle control and cell motility promoting protein associated with highly proliferative breast tumours [294]. Stathmin is involved in regulating microtubules for mitosis [295] and has been shown to be upregulated in breast cancer cells enriched for a metastatic phenotype [296]. Many questions still remain about the significance of stathmin overexpression and it has been hypothesised that since stathmin overexpression sometimes occurs in relation to cell overgrowth, it might be part of a regulatory loop to stop cell proliferation (albeit ineffective in the case of cancer) [294]. If this is the case, it might be an artefact of adherent culture compared to sphere culture. Another protein found to be decreased was anterior gradient protein 2 homolog. This proto-oncogene is involved in differentiation and is associated with oestrogen receptor-positive breast tumours with a potential role in metastatic disease through the regulation of receptor adhesion and functioning [297]. The significance of these proteins’ downregulation in spheres or upregulation in adherent cells is not at the present time fully known.

Interestingly, transgelin-2 was found to have decreased expression on spheres compared to adherent cells. Transgelin-2 is a 22 kDa protein normally found in smooth muscle tissues [298].
A tumour suppressive role of transgelin-2 has been described through its ability to interfere with ERK activation and AP-1 signalling to decrease expression of MMP-9, all molecules involved in cancer invasion [299]. Transgelin-2 has been found to be downregulated in a highly metastatic form of MDA-MB-231 cells compared to the parental line and has been shown to have low expression in lymph node-positive breast cancer tissue [296], suggesting that low expression of this molecule is associated with breast cancer metastasis. The downregulation of this marker on spheres further supports the hypothesis that spheres are enriched for cells with more of a cancer stem cell phenotype than adherent cells and supports the use of tumoursphere culture for investigations related to cancer stem cells.

Proteasome subunit beta type-8 (also called LMP7), a constituent of the immunoproteasome, was found to be decreased within spheres. The immunoproteasome is a multicatalytic complex involved in antigen processing to generate class I binding peptides (involved in antigen processing machinery and cancer reviewed in [300]). Hence, downregulation of the immunoproteasome is an important strategy for tumours to escape from immunosurveillance. Several cancers are characterised by the loss or down-regulation of components of the antigen processing machinery, in particular proteasome subunit beta type-8 [300, 301]. The importance of these findings are somewhat controversial as proteasome subunit beta type-8 proteins have been detected within primary breast carcinoma lesions analysed, but their expression level was found to vary from low to high without any relationship to tumour grading. In cancer, an additional strategy to escape immune surveillance is the substitution of the non-functioning isoform E2 for E1, resulting in immunoproteasome deficiency [301]. At the present time it has not been confirmed whether E1 or E2 isoform was identified. Overall, this finding suggests that cells grown under sphere-promoting conditions might be less immunogenic than adherent cells. A large number of additional experiments would be needed to conclusively confirm that is the case. Included in those tests would be investigating the ability of CD8+ T cells to recognise and respond to presented peptides from both sphere-derived cells and adherent cells. If it is the case that sphere culture produces less immunogenic cells, it might indicate that sphere culture enriches for cells with a phenotype that allows them to evade the immune system, and this is a phenotype that is shared with the putative cancer stem cell.

6.3.2 Proteins upregulated on P8 spheres compared to P2 spheres. The proteome of P8 spheres was compared to the proteome of P2 spheres to determine if any proteins were enriched over serial passage. The growth conditions for P2 and P8 spheres are similar with
regard to everything except passage number. This was not the case for the comparison between adherent cells and sphere culture, as the media for both conditions were dissimilar. Hence, comparisons between P2 and P8 spheres should reliably indicate differences that occur in more similar cells over a time frame in the NSA media. Discussion of the results for molecules that were decreased in P8 compared to P2 spheres are briefly presented here. Several interesting molecules were upregulated in P8 spheres compared to P2 spheres, including some previously discussed in relation to differences between sphere culture and adherent culture.

6.3.2.a Proteins involved in metabolism. P8 spheres demonstrated an increase in expression for several metabolic proteins including fructose-1,6-bisphosphatase. As previously discussed, accumulation of fructose-1,6-bisphosphatase could indicate that cells are utilising glycolytic intermediates above pyruvate kinase to form amino acids, phospholipids, and nucleic acids - in other words, the requirements of highly proliferating cells. This might indicate that serial passage of cells as tumourspheres enriches for cells with a higher proliferative capacity. Also increased was Hsp70, which like Hsp27 (that was found to be enriched on P6 spheres compared to adherent cells) is a stress or heat shock protein that has both anti-apoptotic functions and tumourigenic properties (reviewed in [250]). Also like Hsp27, high Hsp70 expression in breast, endometrial and gastric cancer has been linked to metastatic phenotype, poorer prognosis, and resistance to therapy [254, 302]. Hence, expression of Hsp70 in late passage spheres could indicate enrichment for cells with a more metastatic phenotype. Multiple other molecules involved in the stress response were also found to be upregulated on late passage spheres compared to early passage spheres, including constituents of the proteasome and proteins involved with ubiquitin or ubiquitin-like functions. Additionally, annexin V was found to be upregulated. Annexin V is a marker for apoptosis/necrosis as a result of its high affinity for phosphatidylserine [303]. During the early stages of apoptosis, phosphatidylserine is translocated from the inner side of the plasma membrane to the outer side. Cells undergoing apoptosis are intact cells that can be stained for annexin V. Necrotic cells also stain for annexin V as a result of the leakiness of the plasma membrane. At the present time, it is not known how viable cells from intact spheres are. Trypsinizing spheres to prepare single cells often resulted in a proportion of cells that were detected to not have intact plasma membranes by PI staining. Annexin V increase in later passage spheres could be attributed to an increase in cells undergoing apoptosis compared to early sphere cultures, which might be more viable. If this is the case, the results relating to other stress response proteins also being increased would make sense as the cells attempt to prevent apoptosis from occurring.
6.3.2.b Proteins with a cytoskeleton function. Another protein found to be increased on P8 spheres compared to P2 spheres was gelsolin/brevin. As previously discussed, this protein is involved in cytoskeleton changes, with the possible implication that these changes could alter the motility or metastatic capacity of cells. Other proteins related to the cytoskeleton were also found to be upregulated including tubulin alpha-1C/B/A and Macrophage-capping protein. Macrophage-capping protein is a calcium-dependent actin filament end capping protein of the same family as gelsolin distributed in both the cytoplasm and the nucleus [304, 305]. Macrophage-capping protein has been reported to be overexpressed in breast cancer, ovarian and pancreatic cancer, glioblastoma and melanoma [306-309]. One report knocking down the expression of macrophage-capping protein in a highly metastatic breast cancer cell line (MDA-MB-231) has reported this reduces the invasiveness of cells in matrigel assays [304]. Furthermore, a report looking at differential proteomic expression between a more highly metastatic line of MDA-MB-231 and the parental line (using a method very similar to the one reported here) has shown that macrophage-capping protein is increased within the more metastatic line [296]. These results support the concept that this molecule is important for a metastatic phenotype. Ezrin-radixin-moesin-binding phosphoprotein 50 (EBP50) was also found to be increased. Normally EBP50 is an adapter protein localised to the apical region of epithelial cells [310]. In tumours EBP50 is overexpressed and located in the cytoplasm or nucleus of epithelial cells [311]. EPB50 might play a role in cell proliferation [312]. In human breast cancer samples a positive correlation between EBP50 and oestrogen receptor status (MCF7 cells are ER⁺) and tumour progression has been identified [313, 314]. The cytoskeleton protein keratin 13 was found to be increased on late passage spheres compared to early passage spheres. Keratin 13 is part of the cytoplasmic cytoskeletons of cells and is thought to play a role in breast cancer growth and metastasis. Additionally, keratin 13 is upregulated in luminal epithelial cells (MCF7 cells are of luminal origin) of secretory phase endometrium. Keratin 13 has been shown to be expressed by MCF7 cells and this expression can be modulated by stimulation of the oestrogen receptor by oestradiol or selective oestrogen receptor modulators. Increased expression of keratin 13 within spheres of later passage might therefore be an indication of enrichment for a metastatic phenotype. One molecule that was found to be increased on late passage spheres compared to early passage ones was serpin B5, and this molecule has been linked to a role as a tumour suppressor protein in human mammary epithelial cells with loss of this molecule found most often in advanced breast cancers [315]. The finding that this molecule is increased in later stage spheres does not support our hypothesis that later stage spheres are enriched with metastatic cells.
However, overall these results indicate that P8 spheres might be more enriched for metastatic cells than P2 spheres and supports the use of tumourspheres for investigating breast cancer stem cells.

6.3.2.c Proteins downregulated within late passage spheres compared to early passage spheres. Few proteins were identified as being downregulated on late passage spheres compared to early passage ones. Of the identified proteins, two related to the stress response: hypoxia upregulated protein 1 and 26s protease regulatory subunit 7. As mentioned previously, hypoxia upregulated protein 1 is upregulated in response to stress and hypoxic conditions and was found to be increased by P6 spheres compared to adherent cells. At the present time it is not known how hypoxic sphere culture is compared to adherent culture. It is likely that spheres are not much more hypoxic than adherent cells. Perhaps the increase in this molecule in early spheres compared to adherent cells is related to the stress of the cells in switching culture conditions and by the time of late passage this stress has been reduced as cells have become accustomed to the new culture conditions. Future experiments might be warranted to investigate: (i) to what extent hypoxia occurs in sphere culture; and (ii) what, if any, time course is involved in the expression of hypoxic markers after the start of sphere culture. The finding that 26s protease regulatory subunit 7 is decreased is interesting in light of the fact that decreased proteasome activity has recently been identified for cancer stem cells from glioma and breast cancer [316]. This marker may be an additional way to target or identify cancer stem cells in vivo.

6.3.2.d Galectin-3 expression is increased on late passage spheres compared to early passage spheres. One final protein identified as being increased on later passage spheres compared to early passage spheres was galectin-3. This protein was considered a good candidate for further study as it displays cell surface expression, has a known role in breast cancer progression, and has some known ligands that can be used for experiments.

6.3.3 Galectin-3 expression and blocking within tumourspheres. Galectin-3, a lectin family member, selectively binds β-galactoside residues and consists of a carbohydrate recognition domain, a collagen-like domain, and a NH2-terminal domain. Galectin-3 is located in cytoplasmic, nuclear and extracellular sites [317]. Galectin-3 has an affinity for lactose and LacNAc (Reviewed in [318]). Galectin-3 has several described associations and roles related to
cancer, in particular in relation to cell-cell and cell-matrix interactions, metastasis, angiogenesis, tumour progression and resistance to apoptosis/anoikis (reviewed in [319, 320]). Galectin-3 enhances metastasis through promoting tumour cell adhesion [321, 322], invasiveness [323], and inducing tumour cell proliferation and angiogenesis [324]. Galectin-3 also antagonises tumour cell apoptosis and anoikis [325-327]. In relation to breast cancer, galectin-3 has a described role in enhancing metastatic disease through resistance to the products of inducible nitric oxide synthase and through its bcl-2-like antiapoptotic properties [328]. One study investigating alterations in galectin-3 expression and distribution within tumour cells in a mouse xenograft model has demonstrated that upregulated galectin-3 is correlated with breast cancer progression [329]. These findings indicate that galectin-3 expression is an important regulator of interactions between tumour cells and the microenvironment, and as such galectin-3 is an important marker of tumour cells including potentially cancer stem cells (Figure 6.9).

Galectin-3 is a promising target for cancer therapy. One molecule used as a competitive inhibitor of natural ligands for galectin-3 is the polysaccharide modified citrus pectin (MCP). In vitro MCP has been shown to inhibit aggregation of tumour cells, angiogenesis, and tumour cell adhesion to endothelial cells [324, 330, 331]. These inhibitions should theoretically also inhibit metastasis, and this was subsequently shown to be the case for intravenous and oral administration of this compound in animal models of metastatic B16-F1 melanoma [330], prostate adenocarcinoma [331] and human breast carcinoma cells [332]. Galectin-3 null mice have been shown to be relatively healthy [333], suggesting that inhibition of galectin-3 might be therapeutically valuable while sparing patients from severe side effects. Investigations into inhibitors/ligands of galectin-3 are proceeding in several labs, as is the assessment of natural ligands of galectin-3. We have used LacNAc as an inhibitor to investigate the role of galectin-3 in tumoursphere formation in vitro. Unfortunately, natural saccharide ligands of galectin-3 (such as LacNAc) typically display a low micromolar affinity for galectin-3, and as such many researchers are investigating modifying LacNAc for higher affinity with galectin-3 in particular for use within in vivo models [334, 335]. We have not investigated the effects of galectin-3 inhibition in vivo, instead focusing our investigation on the in vitro model of tumoursphere growth. In the in vitro model LacNAc, is an appropriate choice of ligand for investigation of inhibition of galectin-3 (Figure 6.9).
Figure 6.9 Galectin-3 and LacNac. (a) Extracellular functions mediated by galectin-3. Secreted galectin-3 mediates angiogenesis by facilitating migration, chemotaxis and morphogenesis of endothelial cells. Cell surface galectin-3 is involved with homotypic and heterotypic cell aggregation and invasion leading to metastasis. Extracellular galectin-3 cross-links cell surface glycol-conjugates, forms dimers and multimers, and delivers signals inside the cell. The ∼22 kDa fragment of MMP cleaved galectin-3 binds to the glycan receptors more efficiently than the intact protein, the functions of smaller fragments are not fully known. (b) the structure of N-Acetyl-D-lactosamine (LacNac), a ligand for Galectin-3. (c) X-ray structure of Galectin-3:LacNac complex.

The development of metastatic disease requires both heterotypic tumour cell adhesion to endothelia and homotypic adhesion between tumour cells [336-338]. Galectin-3 has been implicated in the homotypic aggregation of tumour cells during metastasis via bridging with branched, soluble complementary glycoconjugates between cells [339]. This aggregation has been shown to be a result of interactions between galectin-3 and cell surface Thomsen-Friedenriech glycoantigens (TFAg) [340, 341] and/or 90K/Mac-2BP [342]. Homotypic intercellular communication between metastatic cells greatly increases their clonogenic growth and survival [336]; thus blocking this interaction could be a treatment to prevent the early steps of metastatic disease. Several studies related to breast cancer have shown blocking of homotypic adhesion of tumour cells (and heterotypic adhesion to endothelial cells) using ligands of galectin-3 and the subsequent reduction in metastatic disease [332, 343, 344]. We have demonstrated that the use of LacNAc prevents the formation of tumourspheres in a dose dependent manner for MCF7 breast cancer cells but not for MDA-MB-436. MCF7 single cells treated with LacNAc and placed into sphere culture resulted in fewer recognisable spheres formed, as the cells were more likely to adhere to the tissue culture flask than to each other. MDA-MB-436 cells were not affected by treatment with LacNAc. Subsequently, we demonstrated that another cell line (HBL-100) was susceptible to LacNAc disruption of tumoursphere growth, demonstrating that the results in relation to MCF7 were not unique. However, other cell lines were also shown to be unaffected by LacNAc treatment (SUM-159-PT and BT-474). Treatment of MCF7 cells with LacNAc also demonstrated a decrease in galectin-3 cell surface expression for MCF7 cells, but did not alter cell viability or changes in cell cycle. Changes in cell cycle was investigated as galectin-3 has previously been reported in some models to arrest the cell cycle in G1 phase through inhibition of cell cycle regulation [325]. We observed no changes in cell cycle at either 48 hours or 5 days with the addition of LacNAc to the culture.

Galectin-3 expression was found to be increased on P8 spheres compared to P2 spheres using the proteome approach. This change in expression was also observed between P2 and P6 spheres when using a flow cytometry approach. It appears that galectin-3 expression observed in the flow cytometry approach is mostly associated with dead/dying cells. This was evident at P2 where for both MCF7 and MDA-MB-436 there was low expression of cell surface galectin-3 for viable cells from either spheres or adherent cells, but high expression of galectin-3 when looking at dead/dying cells. These cells are permeable to PI and would also be permeable to the antibody directed against galectin-3. For this reason, it is likely that galectin-3 expression observed in
dead/dying cells is due to expression of galectin-3 within organelles or the nuclear membrane. However, galectin-3 cell surface expression of intact viable cells was clearly shown to increase from P2 (MFI around 150) to P6 (MFI around 1000). The expression of this cell surface galectin-3 could be blocked with the addition of LacNAc. The proteome approach and the flow cytometry approach to looking at galectin-3 expression are looking in two different ways at cells in two different states. Flow cytometry requires single cells to be used and this in turn requires trypsinizing and processing the spheres in a way that might lead to loss in cell viability and cleavage or downregulation of cell surface proteins. The advantage of the proteome approach is that whole spheres are used in the processing of the samples without addition of trypsin or mechanical dissociation of spheres. It is thought that the process of passaging cells in the tumoursphere assay results in cell death for around 90% of cells leaving only the growth factor-responsive cells to survive and repopulate the sphere (self-renewal). This is reasonable as for the MCF7 experiment with addition of LacNAc at 48 hours there were not many cells that were clearly PI negative, while five days later there was an increase in viable cells. Observations of galectin-3 expression for MCF7 cells thus indicate that not only is the passage number important (galectin-3 appears to increase with serial passage of cells), but also the time from passage is important (as the tumoursphere assay causes a disruption in spheres at passage that leaves many dead/dying cells in the first 48 hours and more viable cells after). The results of galectin-3 expression within MCF7 tumourspheres are intriguing but leave many questions unanswered.

What is the nature of galectin-3 expression in relation to cancer stem cells or to the malignant LTP? This question has not been properly addressed in these series of experiments. In the case of some cell lines (MCF7, HBL-100) but not others (MDA-MB-436, BT-474, SUM-159-PT) galectin-3 expression and function might be important for tumoursphere formation (and hence on/within LTP) or cell-cell contact that is involved in keeping the sphere together (and hence on/within LTP, STP, or differentiated cells). MCF7 and HBL-100 cells treated with LacNAc do not form spheres as efficiently as cells left untreated. This could be due to the ligand blocking the homotypic adhesion of cells together in such a way as cells are more likely to stick to the tissue culture flask than to each other. Another way that sphere formation could be blocked is by LacNAc preventing the function of LTP cells to self-renew and form spheres. If this is the case then galectin-3 is probably not a universal marker for malignant LTP cells as (i) LacNAc blocking of galectin-3 was not effective for some cell lines; and (ii) galectin-3 expression was not observed in some cell lines that can form spheres such as MDA-MB-436. However, galectin-3 expression was shown to be enriched upon serial passage for MCF7 cells using both proteomics
and a flow cytometry approach. Galectin-3 expression might be important for MCF7 malignant LTP cells or its expression might be enriched in STP or even fully differentiated cells. It is thought that the number of LTP would remain the same throughout sphere culture. STP and differentiated cells are likewise thought to remain the same, as at each passage the previous LTP cells are the sole survivors of passage and can repopulate the sphere with additional STP and differentiated cells. However, it is possible that some STP and differentiated cells survive passage and are then the ones that are enriched on further passage. If this is the case then the enrichment seen in galectin-3 expression over serial passage could be expressed on differentiated cells or STP cells and not the malignant LTP. Further experiments would be necessary to determine this.

These experiments highlight the ability to use proteomics to identify candidate proteins involved in tumoursphere formation/function. It is important to note that further validation of these candidate proteins will need to take into account isolation of individual cell populations within tumourspheres to assess their stem cell-like phenotype in order to conclusively state that they are associated with cancer stem cells. We made use of the tumoursphere assay as a read-out for self-renewal efficiency of cells within tumourspheres. This assay, as previously discussed, is a good in vitro approach to investigating cells with stem cell-like functions. Future experiments will need to address the tumour-initiating properties of cells identified/isolated based on candidate cancer stem cell-like proteins in in vivo models.

6.4 Conclusions

Our results show a proof of principle for using proteomics to investigate differences between protein expression by early and late tumourspheres or by tumourspheres and adherent cells. This approach can be used to identify candidate proteins for further investigations. We have identified a number of proteins enriched within spheres relating to (breast) cancer and stem cell functions. Further investigations into one of these proteins, galectin-3 was performed to show how proteins identified in this manner can be translated into investigations of sphere culture. Further experiments are needed to show reproducibility and also to investigate other cell lines. In particular, this approach would be ideal for investigating the tumoursphere or cancer stem cell phenotype from a variety of different subtypes of cancer (basal/luminal) and relating this to the profile for the different progenitor cells of the mammary epithelium, similar to the approach that was performed by Lim et al. comparing gene expression profiles of mammary epithelial and stromal subsets to the subtypes of breast cancer [39].
Chapter Seven: Irradiation Effect on Tumourspheres and Cell Cycle Analysis of Tumourspheres

7.1 Introduction

Radiotherapy is a vital component of breast cancer treatment implemented as a first-line therapy, for palliative treatment, or to reduce the risk of recurrence following surgery. In the metastatic setting, radiotherapy is a first-line therapy for patients with multiple brain metastases [345] and can provide pain relief from bone metastases [346]. The majority of patients undergoing lumpectomy (breast-conserving surgery) for invasive disease receive radiotherapy [347], as do patients that are not candidates for breast-conserving surgery who elect for a mastectomy [348]. Meta-analysis of 42,000 women in 78 clinical trials demonstrates that radiation therapy after either lumpectomy or mastectomy in women with early-stage breast cancer significantly reduces both five-year recurrence and 15-year mortality rates [349]. Data from clinical trials demonstrates that radiation therapy can prevent breast cancer recurrence and improve overall survival in some patients, but also demonstrates that radiotherapy (as part of a multi-modality treatment) does not cure all patients as radiation resistance occurs [349] (and reviewed in [350]). The 10 year local recurrence rate in cohorts of oestrogen receptor positive or negative patients who received lumpectomy and radiation was 13% compared to 47% for patients who did not receive radiation (Figure 7.1). In the case of patients receiving a mastectomy and radiation, the recurrence rate was 8% compared to 28% not receiving radiation. These analyses imply three things; (i) that radiation therapy prevents breast cancer recurrence in some patients who have radiation sensitive disease and hence benefit from treatment with radiation; (ii) that some patients are not at risk for local recurrence and do not benefit from radiation therapy; and (iii) that radiation resistant disease is present in some patients. Emerging data implicates breast cancer stem cells in radiation resistance (reviewed in [350]).

Numerous factors have been postulated to be associated with cancer stem cells contributing to tumour recurrence post radiation (Figure 7.2). Contributing factors might include the activation of self-renewal of cancer stem cells post radiation or the intrinsic ability of cancer stem cells to survive radiation. The mechanisms by which cancer stem cells are radioreistant and lead to tumour recurrence are not fully elucidated. As such, we have been interested in studying the response of tumourspheres (or the growth of tumourspheres after irradiation of monolayer cells) from a variety of breast cancer cell lines to irradiation. Radiosensitivity/resistance is inherently a
Clinical trials demonstrate that radiation therapy prevents breast cancer local recurrence in some patients while other patients have radiation resistant disease. For patients undergoing lumpectomy, 10 year local recurrence rate for patients receiving radiation is 13% compared to 47% for patients not receiving radiation therapy. For patients undergoing mastectomy these numbers are 8% and 28% respectively. This implies that a number of patients (shaded in yellow) have radiation sensitive breast cancer and receive a benefit from treatment. It implies as well that 53% (100%-47%) of lumpectomy patients and 72% (100%-28%) of mastectomy patients were not at risk for recurrent disease and did not require radiation therapy. Finally, these results also imply that a number of patients have radiation resistant disease.

Factors contributing to recurrence of disease after radiation could be due to properties of tumour-initiating cells (cancer stem cells). Tumour-initiating cells (TICs) and non-tumour-initiating cells comprise a tumour. It has been proposed that radiation causes cell death primarily for non-TICs compared to TICs. TICs may lead to tumour resistance through at least two non-mutually exclusive methods. One method is through undamaged or repaired TICs self-renewing through asymmetric and symmetric division as a response to the radiation. This method might entail the activation of signalling pathways leading to radioresistance. Another method postulated might be the intrinsic resistance of TICs to radiation. Differences between TICs and non-TICs that might lead to this intrinsic ability for TICs to survive radiation might include differences in the balance of reactive oxygen species (ROS) between then two populations, enhanced DNA repair efficiency within TICs, and differences in cell cycle checkpoint regulation.
relative term. As such, we have compared tumourspheres to adherent cells. Furthermore, results from several studies have demonstrated that tumour cell lines respond differently in vitro than they do in vivo and that radiosensitivity in vitro does not necessarily predict the radioresponse in vivo [351-353]. It is certainly an important avenue of research to assess the in vivo effects of irradiation on TICs. However, we have focused on studying only in vitro responses of cells to irradiation for this thesis work.

Radiotherapy is used to kill tumour cells because it can cause DNA damage leading to cell death. This has largely been attributed to the production of hydroxyl radicals generated from DNA, water and other nearby biomolecules by the high-energy radiation. Recently, another method for how DNA damage occurs has been postulated [354, 355]. It has been reported that prehydrated electrons formed by radiation can react with certain nucleotides (dGMP and dTMP) in solution, suggesting that these prehydrated electrons might interact with bases of DNA duplexes forming transient anions within the DNA, which upon decomposition could cause bond breaking and hence DNA damage. As a result, one main criterion for assessing radiation response is to investigate DNA double strand breaks (DSBs) and, importantly, the repair of DNA DSBs. One way to determine this is through assessing γH2AX foci in samples of cells post-irradiation. The histone H2AX becomes phosphorylated to γH2AX at areas of radiation-induced DSBs forming nuclear foci, and this can be visualised through immunohistochemistry or flow cytometry [353, 356]. These foci disperse with time, and the number remaining at 24 hours post-irradiation has been used to show a correlation with cellular radiosensitivity [357, 358]. Another component of radiation-induced DNA damage response is cell cycle checkpoint activation – i.e. cells arrest or fail to arrest at specific stages of the cells cycle. As such cell cycle distribution following irradiation is often reported. Apoptotic cell death has been reported to be an infrequent event after irradiation and has been reported to not account for the reportedly increased radiosensitivity phenomenon of cancer stem cells [359]. Indeed some evidence indicates that apoptosis is not a major mechanism of cell death following treatment with chemotherapy for cancer cell lines and that more relevant endpoints of cell death include the loss or reproductive ability of cells [360]. Other methods used to assess the response of cancer stem cells to radiotherapy include assaying clonogenic survival and increased tumourspheres production following irradiation. One property often attributed to normal stem cells has been their quiescent state, and this has long been thought to be the reason for radiation resistance in cancer stem cells. However, it has now been shown that cancer stem cells do cycle and that quiescence alone cannot be used to exclusively explain the reportedly radioresistant response of cancer stem cells to radiation [361, 362]. As such, the
methods described above have been used to investigate the response of cancer stem cells to radiation.

Considerable effort has gone into the study of cancer stem cells and radiation resistance; however, relatively few studies have shown compelling definitive evidence that cancer stem cells are radioresistant. Indeed, for some cancers such as glioblastoma the radiosensitivity of the cancer stem cell (or tumour-initiating cell) pool is quite controversial. Bao et al. have reported that CD133+ glioblastoma cells (the cells thought to be tumour-initiating cells) are more resistant to radiation compared to CD133- cells, with a reduced level of apoptosis and an increase in colony-forming efficiency following irradiation, attributed to more efficient activation of DNA damage repair and activation of cell cycle checkpoint proteins (chk1 and chk2) in the cancer stem cell pool [363]. However, McCord et al. have shown that when comparing the CD133+ glioblastoma tumour stem cells to established glioblastoma cell lines, the CD133+ cells have a reduced capacity to repair DNA double strand breaks and are radiosensitive [359]. These studies highlight the fact that the response of cancer stem cells to radiation is still controversial and not fully understood.

For the case of breast cancer, a few observations and preclinical reports make an argument for the radiosensitivity of breast cancer stem cells. Clinically, some reports have shown a correlation between tumours without luminal markers of differentiation and radiation resistance, such as oestrogen receptor negative tumours [364] or basal or HER2+ tumours [365]. Other reports have indicated that mammary gland stem cells and cancer stem cells themselves lack luminal differentiation markers (reviewed in [366]). From these observations it could be inferred that cancer stem cells are behind resistance to radiotherapy for some breast cancers. Pre-clinically, in a mouse model of breast cancer, a putative cancer stem cell population was found to be radioresistant compared to tumour cells with a non-stem cell-like phenotype [351]. Only a few preclinical studies have attempted to correlate human breast cancer stem cells with radiation resistance. In one study the radiation effect on breast cancer stem cells was studied in vitro using two breast cancer cell lines (MCF7 and MDA-MB-231), using staining of γH2AX and the measurement of reactive oxygen species (ROS) as functional tests for radiation resistance [90]. This study found that the number of double strand breaks identified by staining of γH2AX was decreased in tumourspheres compared to adherent cells following ionization radiation. The conclusion was that stem/progenitor cells and differentiated cells are damaged equally by radiation in terms of double strand DNA breaks immediately after radiation, but stem/progenitor
cells repair damage more quickly. ROS levels were found to be lower in tumoursphere-derived cultures after treatment with radiation than in adherent/monolayer culture, indicating high intracellular levels of radical scavengers. In MCF7 cells, cancer stem cell/progenitors cultured as tumourspheres were more resistant to radiotherapy than cells in monolayer culture, and fractionated radiotherapy increased the proportion of cells with the CD44⁺CD24⁻/low phenotype and the number of tumourspheres [90]. Fractionated radiotherapy also activated Notch-1 signalling [90]. Notch-1 signalling promotes self-renewal of normal mammary stem cells [367], and thus the activation of Notch-1 post radiotherapy might be a mechanism for accelerated repopulation of tumours during gaps in radiotherapy treatment. A subsequent study has demonstrated using breast cancer cell lines (MCF7 and T47D) that cell populations are enriched for cancer stem cells after irradiation and that tumoursphere forming efficiency is increased over three generations for cells exposed to sublethal doses of irradiation [368]. Lagadec et al. also demonstrated that cancer stem cells (identified in this study as CD44⁺CD24⁻/low and lacking proteasome activity) were resistant to radiation-induced apoptosis and were arrested in the G₂ phase of the cell cycle, while non-cancer stem cells were prone to radiation induced apoptosis and were driven into senescence. They also noted that the frequency of cancer stem cells in G₀ was higher in cells that were not irradiated compared to irradiated, and that radiation mobilised the cancer stem cell pool from a quiescent state to a proliferative one (G₂). These reports make a strong case for breast cancer stem cells playing a role in radiation resistance; however, in each case the studies used only two cells lines. Future studies will benefit from using a larger panel of cell lines capable of growth as tumourspheres in investigating the effect of irradiation on breast cancer cell lines.

Radiotherapy is an integral part of the treatment of breast cancer and a number of investigations have now postulated that breast cancer stem cells are responsible for radiation resistance. Future work remains to clarify the mechanisms of breast cancer radioresistance/sensitivity. Hence, investigating the response of BCSCs to radiation is an important avenue of cancer stem cell and breast cancer research. We hypothesise that tumourspheres contain cells with differences in the cell cycle compared to adherent cells, and that enrichment or resistance of cancer initiating cells (cancer stem cells) occurs in tumourspheres during the course of fractionated irradiation. As such, we have investigated the effects of irradiation on tumourspheres and adherent cells; the cell cycle expression of tumourspheres and matched adherent cells with and without exposure to irradiation; and differences in cell cycle expression for slow cycling cells within tumourspheres compared to all cells. Results indicate that SFE was only increased for MCF7 cells (out of six cell lines tested)
following exposure to irradiation. An increase in the frequency of cells in the G\(_2\) phase of the cell cycle was found following irradiation. Analysis of cell cycle distribution for slow-cycling cells compared to all cells also demonstrated an increase of cells in the G\(_2\) phase of the cell cycle for slow cells compared to all cells. The results we obtained for MCF7 cells are similar to those observed by other researchers [90, 368]; however, we found that the main response elicited by the majority of breast cancer cell line derived tumourspheres to irradiation was not an increase in SFE but rather a decrease. This might indicate that the response of cancer stem cells to irradiation is not a universal feature of cancer stem cells, but rather a function of the cell line used.

### 7.2 Results

#### 7.2.1 Irradiation of cells in monolayer conditions alters the short term growth of adherent and tumoursphere cultures of breast cancer cells.

As an initial screen, five breast cancer cell lines (HBL-100, SUM-159-PT, MDA-MB-436, MCF7, and ZR-75-1) were cultured as adherent cells for five days and then irradiated at either 0, 10 Gy or 5 courses of 3 Gy given every 24 hours. 24 hours after last irradiation, cells were cultured in triplicate in both adherent and tumoursphere-promoting conditions and were screened for their initial growth 5 days after irradiation (experiment performed twice for sphere culture and once for adherent culture with representative results shown here). Additionally, the effect of irradiation on sphere forming efficiency was assessed. Irradiation affected the ability of adherent cells to increase in numbers and culture over the period of five days post-irradiation (Figure 7.3). Sphere culture was also affected by irradiation with a decline in sphere formation over time observed both visually (Figure 7.4) and quantitatively using the sphere forming assay (Figure 7.5) for all cell lines except MCF7. MCF7 demonstrated an increase in spheres following radiation (done at least twice with representative results shown). A one-way ANOVA with a post test for linear trend indicated an increase in sphere formation for MCF7 cells when plated at 5000 single cells per well (p = 0.0011, **). No other cell line screened demonstrated a similar increase in sphere formation post-irradiation and conversely demonstrated a significant decrease, (***, p ≤ 0.0001).

In all cases irradiation adversely affected the recovery of viable cells for further passaging as assessed by triplicate cultures counts of viable cells (Figure 7.6). MCF7 tumourspheres were found to have increased frequency of recovered viable cells following irradiation compared to adherent cells for both the 10 Gy treatment group (*, p = 0.0443) and the 5x3 Gy treatment group...
(**, p = 0.0036). SUM-159-PT tumourspheres at the 10 Gy treatment group demonstrated this increase as well (*, p = 0.0179). No other cell lines demonstrated this result.

7.2.2 Irradiation alters the ability of MCF7 and MDA-MB-436 cells to form tumourspheres at both P1 and P2. MCF7 and MDA-MB-436 cells were chosen for further experiments. Cells from each cell line were irradiated as adherent cells or tumourspheres at 0, 10 Gy, or 5 x 3 Gy. 24 hours later, cells were passaged into tumoursphere-promoting conditions (as either P1 (irradiated as adherent cells) or P2 cells (irradiated as spheres). Six days after last irradiation, cultures were assessed for tumoursphere growth and SFE. Irradiation was found to increase sphere formation for MCF7 cells at both P1 and P2 as assessed both visually (Figure 7.7a) and using the sphere forming assay (Figure 7.8a). Irradiation was found to decrease sphere formation for MDA-MB-436 cells in a dose dependent manner as assessed both visually (Figure 7.7b) and using the sphere forming assay (Figure 7.8b).
Irradiation of breast cancer cells in monolayer conditions alters the short term growth of subsequent monolayer cultures assessed visually. Five breast cancer cell lines were cultured as adherent cells for 5 days and irradiated at either 0, 10 Gy or 5 x 3 Gy. 24 hours after last irradiation cells were passaged and cultured in triplicate in adherent conditions. Cells were screened for their initial growth 5 days after irradiation. Representative 4x photomicrographs are depicted here. Bars represent 100 μm.
Figure 7.4. Irradiation of cells in monolayer conditions alters the short term growth of subsequent tumoursphere cultures assessed visually. Five breast cancer cell lines were cultured as adherent cells for 5 days and irradiated at either 0, 10 Gy or 5 x 3 Gy. 24 hours after last irradiation cells were passaged and cultured in triplicate in tumoursphere-promoting conditions. Cells were screened for their initial growth 5 days after irradiation. Representative 4x photomicrographs are depicted here. Bars represent 100 μm.
Figure 7.5. Irradiation of cells in monolayer conditions alters the short term sphere forming capacity of breast cancer cells. Cells irradiated as adherent cultures and placed into sphere forming assay (n = 10) at either 5000 single cells (white) or 1000 single cells (shaded) plated. A one-way ANOVA with a post test for linear trend indicated an increase in sphere formation for MCF7 cells when plated at 5000 single cells per well (p = 0.0011, ***). When 100 single cells were plated no such effect was seen. No other cell line screened demonstrated a similar increase in sphere formation post irradiation and conversely demonstrated a significant decrease, (***, p ≤ 0.0001).
Irradiation of cells in monolayer conditions alters the recovery of viable cells after subsequent culture in adherent or tumoursphere promoting conditions. Recovery of viable cells following irradiation and culture in triplicate for 5 days as either adherent (ADH) conditions or tumoursphere-promoting conditions (SPH). Recovery is standardised to control cells equalling one. All cell lines demonstrated a decrease in viable cells recovered compared to control (p ≤ 0.005, **). The frequency of viable cells recovered was found to be higher in tumourspheres compared to adherent cells only for the MCF7 cell line at both treatments and for SUM-159-PT at 10 Gy treatment. Assay done once in triplicate for adherent cells and done twice in triplicate for tumoursphere promoting conditions (with representative results shown). Bars represent SEM.
Irradiation alters the ability of MCF7 and MDA-MB-436 cells to form tumourspheres differently. Representative 4x photomicrographs of tumourspheres 6 days after last irradiation and 5 days after the start of the sphere forming assay (or second passage). (a) MCF7 cells that were irradiated as adherent cells and then put into the sphere forming assay (P1) readily formed spheres after irradiation. MCF7 cells that were irradiated as spheres (P2) formed spheres after irradiation, but with an increased amount of debris and single cells. (b) Irradiation grossly affected the ability for MDA-MB-436 cells to form spheres when irradiated as adherent cells (P1) or as spheres (P2). This is especially noticeable for the 5x3 Gy treated group. Bars represent 100 μm.
Figure 7.8. Irradiation enriches sphere forming frequency for MCF7 cells but not for MDA-MB-436 cells. P1 single cells derived from spheres or adherent cells were irradiated as previously described (No Tx, 1x10Gy, or 5x3Gy). Cells were then placed into the sphere forming assay in 384 well plates for culture for 5 days, 5000 cells per well, N = 16. (a) Irradiation was found to significantly increase the capability of MCF7 cells to form spheres at both P1 and P2. (b) Irradiation was found to significantly decrease the capacity for MDA-MB-436 cells to form spheres at both passages. *** represents p values ≤ 0.0005. Bars represent SEM.
7.2.3 Irradiation alters the cell cycle for adherent cells and tumourspheres. Adherent cells or P2 spheres from MCF7 and MDA-MB-436 cell lines were irradiated at 0, 10 Gy or 5 x 3 Gy and 48 hours later assessed for cell cycle characteristics (Figure 7.9). For MCF7 cells, irradiation was found to: (i) decrease the frequency of cells in G1 phase for adherent cells only (***, p ≤ 0.001); (ii) decrease the frequency of cells in S phase (*** for adherent and ** for spheres, p ≤ 0.01); and (iii) increase the frequency of cells in G2 phase (** for both spheres and adherent cells). For MDA-MB-436 cells irradiation was found to: (i) decrease the frequency of cells in G1 phase (*** for both spheres and adherent cells); and (ii) increase the frequency of cells in G2 phase and S phase (** for both spheres and adherent cells). This experiment was conducted one time in triplicate cultures. Statistics were done using a one-way ANOVA with a post test for linear trend. Note for figures 7.9, 7.11, 7.13, 7.14, 7.15, 7.17, and 7.18 plots show percentage of cells in GoG1 (full), S (empty) and G2/M (hatched) phases.

7.2.4 Irradiation increases the frequency of cells in the G2 phase and the G2 phase is increased in slow cycling cells compared to all cells. P2 MCF7 tumourspheres were pulsed with CFDA SE and six days later were irradiated at 0, 2, 5, or 10 Gy. 6 hours later, 24 hours later, and 48 hours later cells were analysed for cell cycle expression by flow cytometry (n = 4) and expression of caspase-3 and γH2AX by flow cytometry (n = 3). CFDA SE analysis was done to analyse the top 5% of slow cycling cells per expression of the highest CFDA SE. A representative workflow for this analysis is shown in Figure 7.10. Confirming previous results, a post test for linear trend following a one-way ANOVA revealed a significant decrease in G1 and S phases after irradiation in a dose dependent manner and a significant increase in the G2 phase after irradiation in a dose dependent manner (Figure 7.11). A similar trend was seen at 6 hours and 24 hours (but not 48 hours – data not shown) when analysing only the top 5% of slowest cycling cells, however these results were not as significant as results looking at the whole population. Interestingly, the G2 and S phases of the cell cycle were found to be increased for the top 5% of slowest cycling cells compared to all cells for each set of matching treatments and times (except for 24 hours 5 Gy treatment, a possible outlier). The expression of caspase-3 and γH2AX expression was altered following treatment with irradiation (Figure 7.12). Importantly, γH2AX was increased following exposure to irradiation at various time points as assessed by a post-test for linear trend following a one-way ANOVA.
Figure 7.9. Irradiation alters cell cycle for adherent cells and for tumourspheres. P2 spheres (SPH) or adherent cells (ADH) were irradiated (control = no treatment, 1x10Gy, and 5x3Gy). 48 hours after the last round of irradiation (7 days culture total) cell cycle for (a) MCF7 cells and (b) MDA-MB-436 cells was assessed for both adherent cells and P2 spheres. Plots show percentage of cells in G1 (full), S (empty) and G2 (hatched) phases (n = 3). Bars represent SEM. (c) A one-way ANOVA with a post-test for linear trend demonstrated that increasing amounts of irradiation changes the cell cycle characteristics of the cells. MDA-MB-436 adherent cells and spheres demonstrated a decrease in G1 and an increase in G2 and S phase following irradiation. Conversely, treatment with irradiation for MCF7 adherent cells resulted in a decrease in G1 and S and an increase in G2. For MCF7 spheres there was a decrease in S and an increase in G2 following irradiation.
Figure 7.10. Representative CFDA SE and Cell Cycle Analysis Workflow. Representative MCF7 P2 tumourspheres pulsed with CFDA SE and six days later irradiated at 10 Gy. 24 Hours post irradiation cells are harvested and stained with PI for cell cycle analysis. (a) PI-width by PI-area to collect singlet cells. (b) PI-width by CFDA SE-area showing the top 5% of slow cycling cells in the box. (c) All cells and (d) top 5% of slow-cycling cells histogram for cell cycle analysis. The first peak represents the modelled G0/G1, then second peak G2/M and the middle area S-phase. Other samples were analysed using this same approach.
Irradiation of MCF7 tumourspheres results a dose dependent increase of cells in G2 and the frequency of cells in G2 is increased in the top 5% of slow cycling cells compared to all cells. (a) P2 MCF7 tumourspheres are pulsed with CFDA SE and six days later are irradiated at 0, 2, 5, or 10 Gy. At 6, 24 and 48 hours later cells were analysed for cell cycle expression by flow cytometry (n = 4). (b) The top 5% of slow cycling cells were also analysed. (c) A post test for linear trend following a one-way ANOVA revealed a significant decrease in G1 and S phases after irradiation in a dose dependent manner and a significant increase in the G2 phase after irradiation in a dose dependent manner (all p values below 0.0008, **). A similar trend was seen at 6 hours and 24 hours when analysing the top 5% of slowest cycling cells, however these results were not as significant (all p values below 0.0487, *). (d) The G2 and S phases of the cell cycle were found to be increased for the top 5% of slowest cycling cells compared to all cells for each set of matching treatments and times (except for 24 hours 5 Gy treatment) (all p values below 0.0289, *). Error bars represent SEM.
Irradiation of MCF7 tumourspheres results altered expression of γH2AX and Caspase-3. Day 6 P2 MCF7 tumourspheres were irradiated at 0, 2, 5, or 10 Gy. At 6, 24 and 48 hours later cells were analysed for expression of γH2AX (a) and Caspase-3 (b) \( (n = 3) \). An increase in γH2AX post-irradiation was noted at each time point (all \( p \) values below 0.0018, **). The trend for caspase-3 post-irradiation was more sporadic. A decrease was noted at 6 hours and an increase at 24 and 48 hours. The level of the control cells expression of γH2AX was found to vary over time. Overall, the expression of caspase-3 declined over time for the control cells and other cells following treatment. Statistics used a post-test for linear trend following one way ANOVA. Error bars represent SEM.
7.2.5 Irradiation of MCF7 and ZR-75-1 tumourspheres results in a dose dependent increase of cells in $G_2$ and the frequency of cells in $G_2$ is increased in the top 5% of slow cycling cells compared to all cells. To further the previous results, P2 MCF7 and ZR-75-1 tumourspheres were pulsed with CFDA SE, and six days later irradiated at 0, 2, 5, or 10 Gy. 24 hours later, cells were analysed for cell cycle expression by flow cytometry ($n = 3$). For MCF7 cells, a post test for linear trend following a one-way ANOVA revealed a significant increase in $G_1$ and a decrease in $S$ phase after irradiation in a dose dependent manner and a significant increase in the $G_2$ phase after irradiation in a dose dependent manner (all $p$ values below 0.0001, ***) (Figure 7.13a). These results were similar to what was previously observed with the exception that $G_1$ phase was found to increase instead of decrease over irradiation. ZR-75-1 tumourspheres showed a significant decrease in $G_1$ and $S$ phase and a significant increase in $G_2$ phase in a dose dependent manner after irradiation (all $p$ values below 0.0001, ***) (Figure 7.13b). Irradiation treatment had less of an effect on the cell cycle for the top 5% of slow cycling cells for either MCF7 (Figure 7.13c) or ZR-75-1 cells (Figure 7.13d). A significant reduction in $S$ phase and a significant increase in $G_1$ phase was noted for MCF7 cells ($p$ values below 0.02, *); however, no other significant trends were noted. Once again, a significant increase in the frequency of cells in $G_2$ phase was noted when comparing the top 5% of slow cycling cells to the whole population for both MCF7 and ZR-75-1 cells. This is most easily demonstrated when comparing the no treatment samples to each other, but was also observed when comparing treatment groups to each other.

7.2.6 Irradiation of BT-474 and ZR-75-1 tumourspheres or adherent cells results in a dose dependent increase of cells in $G_2$ and the frequency of cells in $G_2$ is increased in the top 5% of slow cycling cells compared to all cells. P2 BT-474 or ZR-75-1 tumourspheres or adherent cells were pulsed with CFDA SE and six days later were irradiated at 0, 2, 5, or 10 Gy. Six and 24 hours later, cells were analysed for cell cycle expression by flow cytometry ($n = 6$). A one-way ANOVA with a post-test for linear trend revealed a trend for increasing frequency of cells in $G_2$ phase post-irradiation in a dose dependent manner for BT-474 cells cultured as either adherent or tumourspheres (Figure 7.14). This was more significant at 24 hours than at 6 hours. Increases in $S$ phase and decreases in $G_1$ phase were also observed. The effect of irradiation on the top 5% of slow cycling BT-474 cells did not reveal a trend for changes in cell cycle for tumourspheres; however, adherent slow cycling cells were noted to increase the frequency of cells in $G_2$ post-
irradiation (more significant at 24 hours than at six hours). Similar to what was observed previously, ZR-75-1 tumourspheres demonstrated a trend for increasing frequency of cells in G₂ phase post-irradiation in a dose dependent manner for tumourspheres at 24 hours (Figure 7.15). This was not observed for tumourspheres at 6 hours. Adherent ZR-75-1 cells also demonstrated an increase in G₂ phase post-irradiation. Increases in S phase and decreases in G₁ phase were also observed for ZR-75-1. The effect of irradiation on the top 5% of slow cycling cells revealed a trend for an increase of cells in G₂ phase at 24 hours for spheres and adherent cells. In all cases for BT-474 and ZR-75-1 tumourspheres or adherent cells, the frequency of cells in G₂ phase was found to be significantly higher for the slow-cycling cells compared to the all cells (p ≤ 0.0001, ***), using a t-test for statistical comparison. Staining for γH2AX or caspase-3 did not reveal any discernible effect of irradiation (Figure 7.16). Subsequent experiments confirmed the increase in the frequency of cells in G₂ phase post-irradiation for BT-474 and ZR-75-1 P2 tumourspheres when analysing all cells (p ≤ 0.0001, ***) (data not shown).
Irradiation of MCF7 and ZR-75-1 tumourspheres results in a dose dependent increase of cells in G2 and the frequency of cells in G2 is increased in the top 5% of slow cycling cells compared to all cells. (a) P2 MCF7 tumourspheres are pulsed with CFDA SE and six days later are irradiated at 0, 2, 5, or 10 Gy. 24 hours later cells were analysed for cell cycle expression by flow cytometry (n = 3). A post test for linear trend following a one-way ANOVA revealed a significant increase in G2 and a decrease in S phase after irradiation in a dose dependent manner and a significant increase in the G2 phase after irradiation in a dose dependent manner (all p values below 0.0001, ***). (b) ZR-75-1 tumourspheres treated in a similar manner showed a significant decrease in G1 and S phase and a significant increase in G2 phase in a dose dependent manner after irradiation (all p values below 0.0001, ***). Irradiation treatment had less of an effect on the cell cycle for the top 5% of slow cycling cells for either (c) MCF7 or (d) ZR-75-1 cells. A significant reduction in S phase and a significant increase in G1 phase was noted for MCF7 cells (p values below 0.02, *), however no other significant trends were noted. Error bars represent SEM. (e) A significant increase in the frequency of cells in G2 phase was noted when comparing the top 5% of slow cycling cells to the whole population.
Figure 7.14

Irradiation of BT-474 tumourspheres or adherent cells results in a dose dependent increase of cells in G₂ and the frequency of cells in G₂ is increased in the top 5% of slow cycling cells compared to all cells. (a-b) P2 BT-474 tumourspheres or adherent cells are pulsed with CFDA SE and six days later are irradiated at 0, 2, 5, or 10 Gy. 6 and 24 hours later cells were analysed for cell cycle expression by flow cytometry (n = 6). Error bars represent SEM. (c) Summary of statistics. A one-way ANOVA with a post-test for linear trend revealed a trend for increasing frequency of cells in G₂ phase post irradiation in a dose dependent manner for all cells. This was more significant at 24 hours than at 6 hours. Increases in S phase and decreases in G₁ phase were also observed. The effect of irradiation on the top 5% of slow cycling cells did not reveal a trend for changes in cell cycle for tumourspheres. Adherent slow cycling cells were noted to increase the frequency of cells in G₂ post irradiation (more significant at 24 hours than 6 hours). In all cases the frequency of cells in G₂ phase was found to be significantly higher for the slow-cycling cells compared to the all cells (p ≤ 0.0001, ***), (t-test).
Irradiation of ZR-75-1 tumourspheres or adherent cells results in a dose dependent increase of cells in G2 and the frequency of cells in G2 is increased in the top 5% of slow cycling cells compared to all cells. (a-b) P2 ZR-75-1 tumourspheres or adherent cells are pulsed with CFDA SE and six days later are irradiated at 0, 2, 5, or 10 Gy. 6 and 24 hours later cells were analysed for cell cycle expression by flow cytometry (n = 6). Error bars represent SEM. (c) Summary of statistics. A one-way ANOVA with a post-test for linear trend revealed a trend for increasing frequency of cells in G2 phase post irradiation in a dose dependent manner for adherent cells (6 or 24 hr) and spheres (24 hr only). Increases in S phase and decreases in G1 phase were also observed. The effect of irradiation on the top 5% of slow cycling cells revealed a trend for increase of cells in G2 at 24 hours for spheres and adherent cells. In all cases the frequency of cells in G2 phase was found to be significantly higher for the slow-cycling cells compared to the all cells (p ≤ 0.0001, ***) (t-test).
7.2.7 Irradiation of MCF7 tumourspheres or adherent cells results in a dose dependent increase of cells in G2 and the frequency of cells in G2 is increased in the top 5% of slow cycling cells compared to all cells. To confirm previous results relating to MCF7 cell line, irradiation of MCF7 tumourspheres or adherent cells was conducted. P2 tumourspheres or adherent cells were pulsed with CFDA SE and six days later were irradiated at 0, 2, 5, or 10 Gy. Six and 24 hours later, cells were analysed for cell cycle expression by flow cytometry (n = 6). A one-way ANOVA with a post-test for linear trend revealed a trend for increasing frequency of cells in G2 phase post-irradiation in a dose dependent manner for adherent cells and spheres at six or 24 hours (Figure 7.17). Decreases in G1 phase were also observed. S phase changes post-irradiation demonstrated an increase for spheres (different to previous results) and a decrease for adherent cells. The effect of irradiation on the top 5% of slow cycling cells revealed a trend for increase of cells in G2 at 24 hours for spheres and adherent cells, but not at six hours. The frequency of cells in G2 phase was found to be significantly higher for the slow-cycling cells compared to all cells for spheres at six and 24 hours, further confirming previous results. However, not each condition was found to have a significant increase and importantly 24 hour spheres did not demonstrate a significant difference in frequency of cells in G2 phase between all cells and slow-cycling cells (different to previous results) and for adherent cells at 6 hours but not 24 hours (t-test).

7.2.8 Irradiation of HBL-100 tumourspheres or adherent cells results in a dose dependent increase of cells in G2 and the frequency of cells in G2 is increased in the top 5% of slow cycling cells compared to all cells for adherent cells but not for spheres. In order to investigate a basal-like cell line as well, HBL-100 cells were used (Figure 7.18). P2 HBL-100 tumourspheres or adherent cells were pulsed with CFDA SE and six days later were irradiated at 0, 2, 5, or 10 Gy. Six and 24 hours later, cells were analysed for cell cycle expression by flow cytometry (n = 3-6).
Figure 7.16. Irradiation of BT-474 and ZR-75-1 tumourspheres or adherent cells affect on γH2AX and caspase-3 expression. Day 6 P2 tumourspheres or adherent cells are irradiated at 0, 2, 5, or 10 Gy. 6 and 24 hours later cells were analysed for expression of γH2AX and caspase-3 (n = 3). Error bars represent SEM. No discernible trend was observed.
Figure 7.17. Irradiation of MCF7 tumourspheres or adherent cells results in a dose dependent increase of cells in G₂ and the frequency of cells in G₂ is increased in the top 5% of slow cycling cells compared to all cells. (a-b) P2 MCF7 tumourspheres or adherent cells are pulsed with CFDA SE and six days later are irradiated at 0, 2, 5, or 10 Gy. 6 and 24 hours later cells were analysed for cell cycle expression by flow cytometry (n = 6). Error bars represent SEM. (c) Summary of statistics. A one-way ANOVA with a post-test for linear trend revealed a trend for increasing frequency of cells in G₂ phase post irradiation in a dose dependent manner for adherent cells and spheres (6 or 24 hr). Decreases in G₁ phase were also observed. The effect of irradiation on the top 5% of slow cycling cells revealed a trend for increase of cells in G₂ at 24 hours for spheres and adherent cells but not at 6 hours. The frequency of cells in G₂ phase was found to be significantly higher for the slow-cycling cells compared to the all cells for spheres and adherent cells at 6 and 24 hours, but not for each comparison (t-test).
Figure 7.18

(a) (All Cells)

(b) (Top 5%)

(c) Summary of statistics. A one-way ANOVA with a post-test for linear trend revealed a trend for increasing frequency of cells in G₂ phase post irradiation in a dose dependent manner for adherent cells and spheres (24 hr only). Decreases in G₁ and increases in S phase were also observed. The effect of irradiation on the top 5% of slow cycling cells revealed a trend for increase of cells in G₂ at 24 hours and 6 hours for adherent cells and 24 hours for spheres. The frequency of cells in G₂ phase was found to be significantly higher for the slow-cycling cells compared to the all cells for adherent cells at 6 hours for all conditions and for adherent cells at 24 hours for the No Tx and 2 Gy conditions (t-test). None of the other conditions demonstrated an increase in G₂ phase for the slow cycling cells compared to the all cells.
A one-way ANOVA with a post-test for linear trend revealed a trend for increasing frequency of cells in G2 phase post-irradiation in a dose dependent manner for adherent cells and spheres (24 hour only). Decreases in G1 and increases in S phase were also observed. The effect of irradiation on the top 5% of slow cycling cells revealed a trend for an increase of cells in G2 at 24 hours; six hours for adherent cells; and 24 hours for spheres. The frequency of cells in G2 phase was found to be significantly higher for the slow-cycling cells, compared to all cells for adherent cells at six hours for all conditions, and 24 hours for the No Tx and 2 Gy conditions (t-test). None of the other conditions demonstrated an increase in G2 phase for the slow cycling cells compared to the all cells.

7.3 Discussion

Three main conclusions were drawn from our study of the effects of irradiation on tumourspheres and the cell cycle of tumourspheres: (i) MCF7 cells alone out of the breast cancer cell lines tested demonstrated enhanced sphere forming efficiency post-irradiation and tumourspheres formed were more viable than adherent cells post-irradiation; (ii) adherent cells and tumourspheres demonstrate a dose-dependent increase in the G2 phase (correctly the G2/M phase but herein referred to as G2) of the cell cycle response to irradiation; and (iii) the frequency of cells in the G2 phase (correctly the G2/M phase) of the cell cycle is increased in slow cycling cells compared to all cells irrespective of irradiation. These results demonstrate that tumourspheres from one cell line (MCF7) are likely to be more radioresistant than adherent (monolayer) culture, and this confirms previous results using this cell line for studying the response of BCSCs to radiation. These results also suggest that adherent cells and tumourspheres respond in similar ways to radiation treatment – the trend in increasing the frequency of cells in G2 phase post-irradiation. The finding that slow-cycling cells are enriched in the frequency of cells in G2 phase indicates that this population (a population with some of the characteristics of BCSCs) might be less radiosensitive than the general population, as an extended G2 phase could be used by cells as a mechanism to prolong repair of DNA damage [369]. These findings confirm previous results, extend the field of knowledge of breast cancer stem cells and radiation treatment, and suggest future experiments to investigate the response of BCSCs to irradiation.

In this study we have used several different breast cancer cell lines to test the effects of irradiation of monolayer cells on tumoursphere or monolayer growth (tumoursphere growth is a culture condition that demonstrates an enrichment for BCSCs). Initially, we tested five cell lines for
enrichment of sphere forming efficiency post-irradiation (either one dose of 10 Gy or 5 doses of 3 Gy) of monolayer culture: the luminal lines MCF7 and ZR-75-1, and the basal-like lines HBL-100, SUM-159-PT and MDA-MB-436. Subsequent experiments also utilised the luminal line BT-474. The effect of irradiation on subsequent growth as monolayer culture was a uniform decrease in cells for all cell lines tested. This was substantial at both 10 Gy and five treatment of 3 Gy. Next, we tested the ability of cells irradiated as monolayer cultures to form spheres in tumoursphere-promoting culture. Results indicated that only the MCF7 cell line had enhanced tumoursphere forming efficiency (a measure of self-renewal activity of breast cancer stem cells) post-irradiation. All the other cell lines had severely reduced tumoursphere forming efficiency and tumourspheres that did form demonstrated changes in architecture (appeared as looser association of cells). The tumourspheres formed after irradiation of MCF7 cells, on the other hand, were fairly similar in terms of architecture as non-irradiated cells (refer to Figure 7.4). Subsequent experiments demonstrated that the frequency of viable cells collected for MCF7 cells after irradiation was higher than for the other cell lines (with the exception that SUM-159-PT cells were also able to be collected at a similar level of viability as MCF7). Although no clonogenic assay was performed from these experiments, assessment of viability of cells post-irradiation of monolayer cells demonstrated that subsequent culture of MCF7 cells as tumourspheres (P1) were significantly more viable than as adherent cells for both the 10 Gy and the 5x3 Gy treatment groups. Although this is only one time point it does confirm the findings of Phillips et al. that MCF7 tumourspheres have an increased surviving fraction compared to adherent cells post-irradiation [90]. Other cell lines showed either no difference (MDA-MB-436, HBL-100 and ZR-75-1) or less significant differences than MCF7 (SUM-159-PT). These results prompted us to continue to explore the differences in response to radiation between tumourspheres and adherent cells.

Overall, the dissociation of cells into monolayer culture is known to create more sensitivity to various apoptosis agents. As discussed in chapter five of this thesis, 3D culture conditions create physical differences in the microenvironment and allows for cell-cell contact that can change gene expression. Future experiments should compare the effect of irradiation on cells grown in 3D matrigel systems compared to adherent cells. Especially of interest would be to compare the proteome or gene expression between these lines before and after radiation. As discussed previously, tumoursphere growth is such a 3D culture system, albeit matrix-free. As such, we wanted to investigate the differences in irradiating monolayer cultures to irradiating whole mature (day 5 or day 6) tumourspheres. Additional experiments showed that MCF7 cells could survive
irradiation as either monolayer culture or as tumourspheres, and that both starting conditions resulted in an increase in subsequent sphere forming efficiency. This was not the case for MDA-MB-436 cells that were used as a control, as irradiation of either monolayer culture or tumourspheres resulted in a severe decrease in sphere formation upon subsequent culture. Additional experiments showed that irradiation as either monolayer cells or tumourspheres for other cell lines (BT-474, ZR-75-1, HBL-100) also resulted in a decrease in sphere formation similar to MDA-MB-436 (data not shown). These findings suggest that the response of cells to radiation is specific for the cell line tested and does not vary with culture condition used (monolayer vs. tumourspheres). In other words, these results suggest that radiation resistance is not a specific phenotype of tumourspheres, and hence BCSCs, but rather a cell line specific phenotype. MCF7 cells alone had the ability to withstand radiation treatment and in fact demonstrated an increase in sphere formation post-irradiation, perhaps suggesting that radiation stimulates the self-renewal pathway of MCF7 cells, and offers a possible mechanism for the accelerated repopulation of tumour cells observed post-radiation in some breast cancer tumours.

The results obtained for MCF7 cells were similar in some respects to results obtained by other groups. Phillips et al. have shown that fractionated irradiation (the same doses we used) of MCF7 monolayer cells results in an increase of tumoursphere growth at P1 [90]. We have confirmed these results and extend them to demonstrate that irradiation at P1 (i.e. already as tumourspheres) also increases sphere forming efficiency of these cells at P2. Combined, these results suggest that MCF7 BCSCs can survive radiation with their self-renewal capacity intact, and lends credence to the idea that BCSCs can preferentially survive radiotherapy. The observed enhancement in self-renewal activity of irradiated cells compared to non-irradiated cells could indicate that self-renewal is stimulated by radiation, or that self-renewal is relatively enhanced via the depletion of cells that cannot self-renew and that are lost during irradiation. Further experiments will be needed to assess each possibility. Phillips et al. suggest that self-renewal pathways (Notch) are activated following fractionated irradiation [90]. Future experiments will need to follow up this finding to determine the expression of other signalling pathways post-irradiation. Subsequent experiments by Lagadec et al. have shown that MCF7 tumourspheres have enhanced SFE at P1 following irradiation but that this trend is absent during P2 and P3 [368]. These results are not very meaningful as their control untreated cells also showed a decrease in SFE over passage. We have shown in previous chapters of this thesis that MCF7 cells maintain a similar SFE over serial passage. It would be interesting to follow the SFE post-irradiation over several passages to determine if radiation-induced increase in self-renewal is maintained over several generations (we
have not to date done this experiment). Lagadec et al. have shown with more convincing results that T47D cells do have enhanced self-renewal post-irradiation that is maintained for several generations. We have not to date tested the effect of irradiation on T47D cells. Phillips et al. also used the cell line MDA-MB-231 grown as tumourspheres in their research. We have not been successful at growing this cell line as tumourspheres (despite several attempts) and so have tested the effect of irradiation on a variety of other cell lines. Our finding that the majority of breast cancer cell lines do not have enhanced sphere formation (an assay for self-renewal activity) post-irradiation could indicate that radioresistance is not a universal characteristic of BCSCs, but is rather a cell line specific characteristic. It is of note that the majority of experiments conducted on breast cancer stem cells and radiation resistance have made use of MCF7 cells, a cell line that has been proposed to be radioresistant to begin with, perhaps suggesting that this cell line is not the appropriate model, or at best should be considered a control sample.

Caspase-3 is an important component of the proteolytic cascade activated during apoptosis. This protein is activated by many death signals, including those induced by radiation, and subsequently cleaves a variety of important cellular proteins leading to apoptosis. The MCF7 cell line has previously been reported to be caspase-3 deficient [370], and as such these cells have been shown to fail to undergo morphological nuclear and DNA fragmentation following Bax-induced apoptosis (introduction of a gene promoting apoptosis) [371]. Other reports have further demonstrated that MCF7 cells lack caspase-3 expression and that this is responsible for this cell lines decreased ability to undergo apoptosis following stimulation with other apoptotic stimuli [372]. This lack of caspase-3 expression could partially explain why the MCF7 cell line is less susceptible to irradiation treatment (i.e. have a higher frequency of cells that survive irradiation and have an enrichment in sphere forming cells post-irradiation) than other cell lines tested in our experiments. To test this hypothesis, future experiments might investigate the response to irradiation of MCF7 clones that have been stably transfected with the caspase-3 gene, to see if they have a decrease in viability and sphere forming efficiency post-irradiation. Caspase-3 expression (both protein and mRNA) has been demonstrated for BT-474, ZR-75-1 and MDA-MB-436 [373]; and HBL-100 has also been reported to express caspase-3 [374] (the expression of caspase-3 in SUM-159-PT has not been found to be reported at the present time). These cell lines in our system demonstrated a significant response to irradiation (decreased recovery of viable cells and decreased sphere forming efficiency). This further suggests that at least one reason that MCF7 cells have an increased sphere forming efficiency post-irradiation compared to other cell lines is because of the lack of caspase-3 mediated apoptosis. This is likely a contributing factor to
the use of this cell line in experiments assessing the radioresistant phenotype of breast cancer stem cell populations. It is possible that subpopulation of cells (in other cell lines than MCF7) lack expression of caspase-3. It would be interesting to explore the expression of caspase-3 in sub-populations of cells with a putative breast cancer stem cell phenotype to see if lack of expression of caspase-3 correlates with these markers and with radioresistance or chemoresistance. We assessed active caspase-3 expression of cells lines post-irradiation but did not come to any meaningful conclusions. We did observe some expression of active-caspase-3 for MCF7 cells, and rather contradictory, we found less expression for BT-474 and ZR-75-1 cells. Our strategy for analysing active caspase-3 expression would benefit from more optimising. Once activated, caspase-3 is rapidly turned over and, as such, perhaps time points earlier than six hours would be more appropriate for assessment. Also, this analysis could be done using Western and Northern blotting techniques as has been reported in the literature [372].

As previously mentioned, Phillips et al. have demonstrated that one way that MCF7 tumourspheres are more radioresistant is through repairing double strand breaks faster than differentiated cells and through having higher levels of intracellular radical scavengers [90]. Recently, another mechanism for the enhanced survival of MCF7 post-irradiation has been proposed to be down-regulation of the senescence pathway [375]. Karimi-Busheri et al. have demonstrated that in terms of radiation-induced death, adherent cells undergo replicative senescence (because of a lack of caspase-3 apoptosis), and that tumourspheres do not do this. The basis for this was shown to be due to telomerase activity and low expression of p21, a regulator of cell cycle progression through G1 phase. Karimi-Busheri et al. go on to suggest that similar results might be obtained for other cell lines (or cancer stem cell subtypes of other lines) that have inactivating mutations in the proline-rich domain of p53. p53 mediates cell cycle arrest in G1 phase. Cells with mutant type p53 often lack G1 arrest post-irradiation [376], and hence might have an increase in cells arrested in G2 phase, while cells with wild type p53 arrest in G1 phase. Mutations in p53 have been described for BT-474, SUM-159-PT and MDA-MB-436; while MCF7 and ZR-75-1 have wild type p53 [182]. We decided to next investigate the cell cycle expression of these cell lines post-irradiation.

Irradiation has previously been reported to delay the progression of cells through the cell cycle by blocking the progression of cells from G1 to S phase; inhibition of DNA synthesis during S phase; and delaying the progress of cells from G2 phase to mitosis [377, 378]. As a first investigation, we conducted experiments comparing the changes in cell cycle response of adherent cells or tumourspheres to fractionated irradiation using MCF7 and MDA-MB-436 cell lines. We found that irradiation alters the cell cycle of adherent cells and spheres in a similar manner. Post-
irradiation (48 hours for these sets of experiments), for both cell lines and both culture conditions, there was a pronounced increase in cells in the G2 phase of the cell cycle that was dose dependent. G1 (correctly G0/G1 phase but herein referred to as G1) and S phase were found to decrease for MCF7 cells (not significant for MCF7 spheres) while G1 decreased and S phase increased for MDA-MB-436 cells post-irradiation. This increase of cells arrested in the G2/M phase of the cell cycle was interesting in light of other reports of similar findings for cancer stem cells from either glioblastoma [359] or breast cancer [368]. Differences in the distribution through the cell cycle could explain differences in radiosensitivity. Cells in S phase have long been considered to be more radioresistant than cells in other phases of the cell cycle [359]. Interestingly, the frequency of control cells in S phase was fairly similar for MCF7 adherent cells and MDA-MB-436 spheres and adherent cells, and was higher than the frequency for MCF7 spheres. As MCF7 tumourspheres have been shown to be the most radioresistant of these different groups, this might suggest that radiosensitivity cannot be simply attributed to differences in cell cycle distribution (particularly frequency of cells in S phase). These findings warranted further research into cell cycle changes post-irradiation for cancer cell lines.

A more thorough investigation into the effect of irradiation on MCF7 tumourspheres was conducted using CFDA SE pulsed P2 spheres irradiated at 0, 2, 5, or 10 Gy and analysed for cell cycle expression at 6, 24, or 48 hours. Confirming previous results, MCF7 tumourspheres demonstrated a decrease in S and G1 phases and an increase in G2 phase in a dose dependent manner post-irradiation. This was observed at 6, 24, and 48 hours (confirming the last experiment at 48 hours). We subsequently confirmed that this trend occurred also for ZR-75-1, BT-474 and HBL-100 tumourspheres, with particularly convincing results for an increase in G2 phase post-irradiation in a dose dependent manner at 24 hours (but also occurring at 6 hours for some samples). Testing matched adherent cells treated under the same conditions also demonstrated an increased in G2 phase for these cell lines. These results could indicate that 24 hours is a better time point to assess changes in cycle compared to 6 hours. Overall, these results indicate that spheres and adherent cells generally respond to irradiation in a similar way – an increase in cells in G2 phase. This was interesting in light of the fact that the cells had an enrichment in G2 phase of the cell cycle despite p53 mutation status. We expected those cell lines with p53 mutations (Sum-159-PT, MDA-MB-436, BT-474) and MCF7 (because of lack of caspase-3) to accumulate in G2. ZR-75-1 with wild type p53 also demonstrated this trend. It is possible that this line also has some additional defects that prevent cell cycle arrest in G1 phase and this should be further explored in future experiments. It is possible that these results could be exploited to better target
cancer stem cells (and indeed differentiated cells) through developing drugs that target cancer cells in the G₂ phase [379, 380]. Others have reported that an increase in cells in G₂ phase has been attributed to cells that were irradiated during S phase that failed to undergo S-phase arrest [381]. Future experiments should evaluate the activation of G₂ cell cycle checkpoints to further differentiate mitotic cells from cells arrested in G₂. During this experiment we also checked γH2AX expression. An increase in γH2AX was noted post-irradiation at the time points checked (6, 24, 48 hours). However, the expression of γH2AX was found to vary over time for the control cells as well. Additional results assessing changes in this protein's expression following irradiation of BT-474 or ZR-75-1 cells also did not demonstrate any important changes. However, determination of γH2AX was likely not done optimally. Further experiments might benefit from conducting the determination of γH2AX foci at earlier time points such as 5 minutes and 60 minutes post-irradiation and up to 2 hours or 6 hours later. The 60 minute time point was used by Phillips et al. to show that post-irradiation monolayer culture had more DSBs than tumoursphere culture [90]. Future experiments investigating the effects of irradiation on tumourspheres should incorporate the use of these earlier time points to properly assess the DSBs resulting from irradiation.

Activation of the cell cycle checkpoint at the G₂/M boundary allows cells additional time to repair DNA damage caused by radiation, chemicals or faulty replication before mitosis occurs. If repair does not occur the cells will undergo apoptosis. As such, it is important to assess if certain subpopulations of cells are enriched in the frequency of cells in G₂ phase, thus indicating one way that they can overcome radiation-induced apoptosis. Recently, Harper et al. have demonstrated that cells with a stem cell-like phenotype (identified as CD44+ and including the MCF7 cell line) have an extended G₂ phase of the cell cycle and that this is associated with increased apoptosis resistance [382]. We did not find a difference in the frequency of cells in G₂ phase of the cell cycle between adherent cells and spheres. We did, however, find an increase in this frequency within cells that are slow-cycling compared to all cells. This is interesting because it indicates that slow-cycling cells might have an intrinsic ability to repair DNA damage better than all cells. As part of our experiment, we investigated the effect that radiation has on slow-cycling cells in addition to all cells. Interestingly, a similar increase in G₂ phase of the cell cycle was also observed for the slow-cycling cells post-irradiation. In these experiments slow-cycling cells are the top 5% of cells that have not diluted CFDA SE expression. Slow-cycling cells were also found to have more cells in S phase and G₂ phase, and fewer cells in G1 phase than all cells. This result is especially noticeable for the no treatment control cells at either 6 or 24 hours. This might
indicate that slow-cycling cells respond to radiation differently because of their distribution in the cell cycle. As previously mentioned an increase in the frequency of cells in S phase has been associated with radioresistance. The findings from this experiment might indicate that slow-cycling cells are relatively more radioresistant than cells in the greater population. Future experiments should be initiated to answer this question, via sorting populations of slow-cycling and faster-cycling cells and exposing them to irradiation to test self-renewal and survival as well as other tests such as assessment of apoptosis and clearance of γH2AX. This increase in cells in $G_2$ phase might indicate that slow-cycling cells can repair DNA damage better than cells in the faster-cycling populations.

Results investigating the increase in $G_2$ phase for slow-cycling cells demonstrated that adherent cells and spheres derived from BT-474, ZR-75-1 and MCF7 had an increase as expected at each time point compared to the entire population. HBL-100 adherent cells also demonstrated an increase in cells in $G_2$ phase for slow-cycling cells compared to all cells. Interestingly, HBL-100 spheres did not demonstrate this. HBL-100 spheres have a large proportion of cells in S phase and this was true for both the entire population and the slow-cycling population. These results demonstrate that generally slow-cycling cells are delayed in their progression through $G_2$ phase. There was not much difference in the frequency of cells in $G_2$ phase for adherent cells or tumourspheres, demonstrating that this increase in $G_2$ phase is a characteristic of slow-cycling cells and not necessarily BCSCs. Slow-cycling cells also demonstrated a dose dependent response to irradiation for some conditions. For BT-474 cells, this was only observed for adherent cells at six or 24 hours. For MCF7 cells, this was observed for adherent cells at six and 24 hours and spheres at 24 hours. For ZR-75-1 cells, this was observed for spheres or adherent cells at 24 hours only. For HLB-100 cells, this was observed for adherent cells at six and 24 hours and spheres at 24 hours only.

Future studies will be needed to assess the tumorigenicity of cells that have survived irradiation from both adherent cells and tumourspheres to determine the functional outcome of irradiation on cells. This will be important for cell lines, such as MCF7, that we have demonstrated have an enhanced capacity for initial self-renewal (sphere formation) in vitro post-irradiation. Future studies will need to assess the longer term culture of these irradiated cells. Future studies would also benefit from assessing the capability of additional BCSC lines to having enhanced in vitro self-renewal post-irradiation and importantly assessing if clinical breast cancer samples contain
stem-cell like cells that have these characteristics. Determining the phenotype and function of these cells could have important implications leading to the improved treatment of breast cancer.

7.4 Conclusions

The results presented here demonstrate three important findings about breast cancer stem cells (modelled by tumoursphere growth) and irradiation: (i) breast cancer stem cells are not universally resistant to radiation; (ii) breast cancer stem cells respond to irradiation in a similar manner as adherent cells, with an increase of cells arrested in G2 phase of the cell cycle; and (iii) slow-cycling breast cancer stem cells demonstrate enhanced frequency of cells in G2 phase of the cell cycle compared to the entire population of cells. These results confirm some previous research into the field of BCSCs and radiation, and they have important implications in the study of BCSCs and future therapy targeting BCSCs. The findings that breast cancer stem cells are not universally resistant to radiation, and that breast cancer stem cells respond in a similar way to radiation as adherent cells, might indicate that radiation would be an effective treatment for some breast cancers (as it is already known that radiation in general results in prolonged disease-free survival) and could result in killing of some breast cancer stem cells as well as differentiated cells. In our model system, only one out of six cell lines demonstrated an enhanced response to irradiation treatment (MCF7 cells). Determining why this cell line had this response while other cell lines did not would be an important avenue of research and could help determine clinically which patients should receive radiation therapy. The finding that slow-cycling breast cancer stem cells demonstrate enhanced frequency of cells in G2 phase of the cell cycle, compared to the entire population of cells, might also be of interest to the stem cell and cancer stem cell research community. This finding demonstrates that breast cancer stem cells do contain cells that are quiescent as has typically been thought. Instead, they contain cells that are actively engaged in cell cycling. As such, targeting of cells with radiotherapy and chemotherapy, perhaps targeting cells in the G2/M phases of the cell cycle, or changing the cycling of cells out of G2 so that apoptosis can occur, could lead to elimination of BCSCs and improvements in clinical responses.
8.1 Introduction

Despite recent advances in diagnosis and treatment, breast cancer is still a leading cause of cancer-related deaths among women, with as many as 40% relapsing with metastatic disease [383]. Breast cancer patients typically develop brain metastases after metastatic disease involving the bone, liver and/or lung [384]. The risk associated with central nervous system (CNS) metastases varies according to the stage at initial diagnosis, being 2.5% for patients with localised disease, 7.6% for patients with regional disease, and 13.4% for patients with stage IV disease [385]. Previously, brain metastases occurred in late stages of breast cancer, when mortality and morbidity were more associated with non-CNS sites. However, with the improvement of systemic therapies there is now concern that the incidence of symptomatic brain metastases will increase, and as a result control of CNS metastases is now a more important component of patient treatment. Risk factors for brain metastases include an association with younger age, the presence of p53 mutations, oestrogen receptor (ER) negativity, and epidermal growth factor receptor 1 (EGFR) and 2 (HER2) expression [385-387]. Mean 1-year survival following diagnosis of brain metastases varies but has been estimated at 20% [388, 389].

Treatment options for breast cancer brain metastases include the use of corticosteroids to reduce oedema and provide relief of symptoms, whole brain radiation and stereotactic radiosurgery, and surgery with adjuvant radiotherapy [390]. Chemotherapy has found limited use against CNS metastases because of its poor ability to penetrate the blood brain barrier (BBB) or the blood tumour barrier (BTB) (reviewed in [391]). However, there is some evidence that the BTB is more permissive than the BBB in allowing cytotoxic agents to penetrate [392]. Patients with central nervous system metastatic breast cancer treated with trastuzumab (Herceptin; Genentech, CA, USA), a recombinant humanised monoclonal antibody against HER2, have been reported to have objective clinical responses (34% for those whose tumours amplified HER2 and 7% for those whose tumours did not, resulting in a median time to progression of 4.9 months and 1.7 months respectively) [393]. This indicates that systemic therapies that specifically target a protein involved in the pathogenesis of cancer have the ability to control the course of metastatic disease.
HER1, 2, 3, and 4 comprise the epidermal growth factor receptor tyrosine kinase family. Activation causes hetero or homo-dimerization of the receptors followed by phosphorylation of specific tyrosine residues, stimulating signaling cascades mediated by Akt and MAPK (amongst others) and the regulation of cell proliferation and survival (Figure 8.1) [394]. The product of the HER2/neu (ErbB2) oncogene is overexpressed in some subtypes of breast cancer and is associated with a poorer prognosis [95, 96]. HER2 overexpressing breast cancers can be treated with trastuzumab [395]. Single-agent trastuzumab response rates range from 12 to 34% for metastatic breast cancer and show improvements in survival rates for HER2-overexpressing patients treated in the adjuvant setting [228]. Despite these successes, there is a subset of patients who either do not respond to trastuzumab treatment or acquire resistance. Trastuzumab is thought to have several mechanisms for its mode of action and mechanisms for the development of resistance to trastuzumab have also been described (reviewed in [228, 229]). One mode of action is through interfering with extracellular homo and hetero-dimerization of HER2 family receptors, and through downregulation of cell surface HER2 expression through trastuzumab-mediated endocytosis and degradation of the HER2 receptor with the result being inhibition of downstream PI3K and MAPK signalling cascade [228]. Trastuzumab has a described antiangiogenesis role [396]. Other mechanisms that contribute to trastuzumab efficacy include antibody-dependent cellular cytotoxicity, in particular a role for the action of tumour-associated natural killer cells has been described, and suppression of antiapoptotic pathways [230]. Other studies have shown that trastuzumab can increase DNA strand breaks, cause cell cycle perturbations and can cause inhibition of cell growth and arrest in the G1 phase of the cell cycle [226, 231]. Resistance to trastuzumab has also been reported with the majority of patients with metastatic breast cancer initially responding to treatment developing disease progression with 1 year [397, 398]. A variety of mechanisms have been put forth for why resistance develops including: (i) increased circulating extracellular domain forms of HER2 binding to trastuzumab; and (ii) trastuzumab not disrupting the interaction between HER2 with other receptors such as EGFR and hence allowing downstream signalling to be partially restored (reviewed in [228]). As such, other molecules and combinations of drugs have been investigated to circumvent resistance to trastuzumab therapy. Lapatinib (a dual tyrosine kinase inhibitor that interrupts the EGFR and the HER2 growth receptor pathways) has shown both in vitro and in vivo activity, demonstrating growth arrest and apoptosis of in HER2-overexpressing lines, through inhibiting downstream MAPK and Akt activation [399]. The combination of lapatinib and trastuzumab has been found to be synergistic in vitro [400].
Figure 8.1. The HER2 signalling pathway. Ligand binding induces HER2 homodimerization or heterodimerization. This figure illustrates a HER2-HER3 heterodimer. Ligand binding induces dimerization, leading to activation of the intracellular tyrosine kinase. On auto- and cross-phosphorylation of the receptor complex, downstream effectors are recruited leading to MAPK and Akt activation and ultimately proliferation, cell cycle progression or survival.

FKHR, forkhead in rhabdomyosarcoma; Grb2, growth factor receptor-bound protein 2; GSK-3, glycogen kinase synthase-3; MAPK, mitogen-activated protein kinase; mTOR, molecular target of rapamycin; PI3K, phosphatidylinositol 3-kinase; PTEN, phosphatase and tensin homologue deleted on chromosome 10; SOS, son-of-sevenless guanine nucleotide exchange factor.
Interestingly, the mechanisms of trastuzumab resistance may have a relationship to breast cancer stem cell biology (as recently reviewed in [229]). Mechanisms of resistance that have been linked with stem cells include: (i) activation of Notch signalling, which is thought to be important for self-renewal and proliferation of stem/progenitor cells [401]; (ii) induction of epithelial-mesenchymal transition [402]; (iii) activation of pro-survival signals such as survivin and telomerase; (iv) signalling through alternative pathways such as CXCR4 that have a described role for breast cancer stem cells [91]; and (v) blockade of extracellular trastuzumab binding site through molecules such as CD44 that have a described role for breast cancer stem cells [68]. The connection of therapy resistance to stem cell biology further highlights the importance of cancer stem cell research for breast cancer research. Although novel strategies and new drugs are being developed to address the problem of resistance with trastuzumab therapy, this agent still remains an important component of patient treatment.

After the introduction of trastuzumab treatment, an apparent increase in CNS metastases was retrospectively noted amongst HER2+ patients treated with trastuzumab [403, 404]. Clayton et al. investigated the incidence of cerebral metastases in metastatic breast cancer treated with trastuzumab and found that 25% of patients developed cerebral metastases during the course of treatment [404]. Bendell et al. similarly found that 34% of patients developed central nervous system metastases during the course of treatment with trastuzumab (either alone in combination with chemotherapy) [403]. An increase in brain metastases has been associated with HER2-positive breast cancers treated with trastuzumab, likely due to inherent biological properties such as longer survival allowing for progression of the disease (i.e. “unmasking” of the disease in patients who previously would not have had clinical symptoms prior to death), predilection of HER2-positive tumours to develop brain metastases, and treatment-related factors such as the poor ability for trastuzumab to cross the BBB (reviewed in [385]). The influence of the phenotype of the breast cancer has a direct bearing on brain metastases. HER2 overexpressing breast cancer has clearly been linked to central nervous system metastases. However, other clinical classifications/phenotypes of breast cancer have also been found to develop central nervous system metastases.

Basal-like tumors are characterised as high grade, negative for ER, progesterone receptors (PR) and HER2 (they are considered “triple negative” tumours) [71, 405]. Basal-like tumours express similar genes as those of basal/myoepithelial cells of the breast and have characteristics similar to
tumours arising from BRCA1 germline mutation carriers [405]. As a result of being triple negative, the current dogma is that these tumours are unlikely to respond to treatment with trastuzumab or endocrine therapy. A proportion of patients with basal-like tumours present with distant metastases, particularly to the brain [406, 407]. Patients with basal-like tumours were more likely to present with metastatic brain disease (20/11, (18%) vs. 3/178, (2%); p < 0.0001) than patients with non-basal-like tumours, and a basal-like phenotype has been associated with a more aggressive disease (median survival of 10.1 months vs. 25 months, p < 0.0001) [407]. These findings highlight the importance of conducting research for treatment of breast cancer brain metastases of the HER2-negative/low phenotype. The basal-like breast cancer cell line MDA-MB-231 has been extensively used in model systems of brain metastases [390].

Brain metastases of triple negative or basal-type breast cancers have been found to disrupt the BBB, while HER2-positive breast cancers preserve the BBB [408]. Some evidence has shown that trastuzumab cannot pass through the BBB and hence might reduce the effectiveness of this approach for treating HER2-positive breast cancer [409], and this is a problem that subsequent studies will need to address. However, a recent study has shown that trastuzumab-based therapies benefited a group of HER2 negative patients [410]. Additionally, another study has suggested that the cohort of patients who should be given trastuzumab therapy be increased to include those patients with metastatic disease that might be HER2-negative/normal but who express transmembrane regulators of HER3, neuregulin [411], as HER2/HER3 heterodimerization has been linked to tumour growth [412]. Finally, as HER2 has been regarded as a potential breast cancer stem cell antigen [85] it is conceivable that rare cancer stem cells harboring expression of HER2 in a heterogenous tumour that is overall classified as HER2 negative might be responsible for tumour growth or metastasis and hence targeting these cells would be desirable. We hypothesize that trastuzumab treatment may find use in treating triple-negative or HER2-negative/normal breast cancer brain metastasis which express a low level of HER2 by blocking low level HER family receptor dimerization. We provide evidence that the inhibition of HER family receptors through inhibition of HER2 using trastuzumab, even in the absence of HER2 amplification (i.e. triple negative or HER2 non-overexpressed cancers) inhibits tumour formation in an intracranial model of breast cancer and could play a significant role in the management of patients with breast cancer brain metastases.
8.2 Results

8.2.1 Trastuzumab treatment *in vivo* resulted in a decrease in tumour size following injection of MCF7 adherent cells and no tumours following injection of tumourspheres. SCID/Beige mice were injected with 200,000 cells and either treated with 30 mg/Kg trastuzumab intraperitoneally or injected with PBS on day 0. MRI images of week 10 control tumour and trastuzumab treated tumour are shown in Figure 8.2a and 2b respectively. Tumour size of day 23 and day 36 MCF7 adherent and tumoursphere-derived cells were measured and revealed that treatment with trastuzumab resulted in smaller tumours at those time points for adherent cells or absence of tumours for tumourspheres (Figure 8.3a). Day 36 adherent MCF7 tumours were found to be significantly smaller in treated animals, unpaired t-test $p = 0.0241$, *. Overall Adherent MCF7 tumours from treated animals were found to be significantly smaller than control animals, unpaired t-test $p = 0.0201$, *. Since no tumours formed in the tumourspheres + trastuzumab group, a contingency plot comparing presence/absence of tumour for treated and untreated tumourspheres was conducted. A Fisher’s exact test demonstrated a significant difference between the formation of tumours in the control animals and the lack of tumours in the treated mice, $p = 0.009$, **. Compared with tumours originated from adherent cells, tumoursphere-derived tumours were found to be smaller, unpaired t-test $p = 0.011$, *. H&E staining of tumour samples revealed tumours with similar morphology between the tumoursphere-derived and adherent-derived tumours (Figure 8.2c and 8.2d respectively).

8.2.2 Trastuzumab treatment *in vivo* resulted in a decrease in tumour size following injection of MDA-MB-231 cells. In order to determine if another breast cancer cell line that lacked HER2 expression could be treated with trastuzumab, 200,000 MDA-MB-231 cells were administered into the right brain hemisphere sub ventricular zone of SCID/beige mice. Trastuzumab was injected intraperitoneally on the same day as the cerebral injection. Control group mice developed tumor with a mean size of 327 mm$^3$ by week 10. Mice treated on the day of injection with intraperitoneal trastuzumab had smaller average tumour size (72 mm$^3$) compared to controls; unpaired t-test on log transformed data $p = 0.0122$, * (Figure 8.3b).
Figure 8.2. Magnetic resonance images and H&E stains of tumours grown in the brain. (a-b) Magnetic resonance images of week 10 MDA-MB-231 tumours from (a) control mice and (b) trastuzumab treated mice. (c-d) H&E images of day 36 MCF7 tumours from (c) mammosphere-derived and (d) adherent-derived tumours. H&E stains generated with the help of Leonard da Silva, UQCCR.
Figure 8.3. Trastuzumab treatment of HER2-non-overexpressed tumours. (a) Day 23 and day 36 tumour sizes for MCF7 tumours from spheres and adherent culture. No tumours were found in the trastuzumab treated group for sphere injections. Tumour sizes were found to be significantly different for day 36 adherent cells between the no treatment (No Tx) group and the trastuzumab treated group, $p = 0.0241$. (b) Untransformed and log transformed tumour sizes for MDA-MB-231 No Tx and trastuzumab treated groups at week 10. No Tx tumour sizes were found to be significantly larger than the trastuzumab treated group using log transformed data, $p = 0.0122$. 

Figure 8.3

(a) Spheres

(b) Adherent

(a) Untransformed

(b) Log Transformed
Neurons
Astrocytes
Microglia

Neuregulin produced by cells of the brain causes HER2 HER3 dimerization. Trastuzumab may, in addition to its role in inhibiting HER2 homo-dimerization, inhibit the neuregulin/HER3/HER2 signalling axis present in breast cancer brain metastases, and prevent growth of tumours.

Extracellular effects of trastuzumab
- Interference with homo and heterodimer formation between HER-family receptors
- Antibody-dependent immune mechanisms
- Inhibition of HER2 extracellular domain cleavage

Intracellular effects of trastuzumab
- Apoptosis
- Decreased cell proliferation
- HER2 downregulation, dephosphorylation
- Modulation of downstream signal pathways
- Decreased VEGF production
- Potentiation of Chemotherapy

Figure 8.4. Breast cancer brain metastases and the brain microenvironment. Neuregulin produced by cells of the brain causes HER2 HER3 dimerization. Trastuzumab may, in addition to its role in inhibiting HER2 homo-dimerization, inhibit the neuregulin/HER3/HER2 signalling axis present in breast cancer brain metastases, and prevent growth of tumours.
8.3 Discussion

At present, metastatic breast cancer disease is incurable, although many patients now live considerably longer than they previously did with their disease under control. Radiotherapy, specialist surgery, and systemic therapies such as endocrine, cytotoxic chemotherapy, and targeted biological therapies have all played a role in treatment of advanced breast cancer. In the case of metastatic breast cancer, quality of life has been linked to treatment responses [413], as such the discovery of new treatment options or the implementation of old treatment options in a set of patients previously excluded to those options are important aspects of research. Our interest in metastatic breast cancer has thus been in understanding: (i) the role of breast cancer stem cells in metastatic disease; (ii) the “seed and soil” of breast cancer brain metastases, in particular the role of HER family proteins and their ligands; and (iii) investigating the use of trastuzumab therapy for HER2-neagative/low tumours, a category of tumours that have not typically seen much use of this therapy in the clinical setting.

Breast cancer metastases to the brain follows the principles of “seed and soil” outlined by Stephen Paget [414]. Paget analysed 735 fatal cases of breast cancer, complete with autopsy, as well as many other cancer cases from the literature and argued that the distribution of metastases was not due to chance. This postulate gives importance to both the nature of the cells metastasising and the nature (microenvironment) of the organs they metastasise to. In accordance with this finding subsequent research using animal models has demonstrated that particular sets of genes expressed by breast cancer cells influence the colonisation of specific distant sites such as bone, lung and brain [415-418]. We have previously found that a sample from a patient with breast cancer brain metastasis could be grown in tumoursphere conditions for several passages, and have hypothesised that brain metastases might be initiated by or enriched for breast cancer stem cells as assessed by tumoursphere culture. Our interest in breast cancer stem cells led us to investigate breast cancer brain metastases using both adherent/differentiated cells and tumourspheres. However, we were in particular interested in investigating the seed and soil of breast cancer metastases to the brain with an emphasis on investigating HER2-negative tumours – the “seed.” Two HER2-negative/low breast cancer cell lines were used in this investigation and both were capable of growth in the brain microenvironment of immunocompromised mice – the “soil.” Additionally, one of the cell lines was also grown as tumourspheres and tumour growth in the brain was shown to be morphologically similar to tumours derived from adherent cells.
Tumoursphere-derived cells were capable of growth in the mouse model, although to a lesser degree than the adherent population. This might be due to such factors as the overall higher viability of the adherent population compared to the tumourspheres. The tumourspheres were treated more roughly upon processing into single cells and although at the time of checking cell viability, equal numbers of viable cells were injected, it is possible that single cells from the tumourspheres actually had less viable cells than realised over time. It is also possible, that the tumoursphere-derived cells are equally as tumourigenic as the adherent cells in serial passage and just not so upon the first injection into mice, although this experiment has not been performed. It would be interesting to analyse the serial passage of tumoursphere-derived cells and adherent cells in an intracranial model to assess whether tumoursphere culture enriches for cells capable of establishing tumours over serial passage in this model. Importantly, we have shown that both cells grown as tumourspheres and as adherent conditions are capable of establishing tumours in the mouse brain microenvironment and both show response to treatment with trastuzumab.

We found that two HER2-negative/low breast cancer cell lines injected into the brain could be treated with trastuzumab therapy to prevent or delay tumour progression. For future studies it would be beneficial to also include an irrelevant isotype-matched antibody as the control instead of PBS injected animals as was used in this study. Evidence suggests that trastuzumab does not cross the blood brain barrier well, even in the case of the presence of metastatic disease [409]. Despite the poor ability to cross the BBB (trastuzumab has a molecular weight of 148,000 Kd) trastuzumab might play a role in treatment of breast cancer CNS metastases, even in the case of HER2-negative tumours. As previously mentioned, basal tumours often disrupt the BBB and thus HER2-negative/low tumours (such as basal ones) might be better able to respond to trastuzumab therapy as trastuzumab would now be able to better cross the BBB. Other ways to get around the problem that the BBB imposes include use of intracerebral microinfusions of drugs such as trastuzumab directly into the brain via the use of an intracerebral cannula or the use of other small molecule inhibitors of HER family proteins that are better able to cross the BBB than trastuzumab, possibly in combination with chemotherapy and radiation (reviewed in [419]). Initially we hypothesized that trastuzumab treatment might target cancer stem cells expressing HER2 in vivo and in vitro. In chapter 5 of this thesis, however, no difference was noted in sphere forming efficiency for trastuzumab treated breast cancer cell lines (including HER2 over-expressing cell lines and HER2 normal cell lines) compared to controls. This does not definitively rule out trastuzumab targeting rare HER2 expressing cells in our “negative” cell lines in vivo in the results presented in this chapter, but it also does not suggest that this is what is occurring. We
propose that alternatively what is occurring is that blocking even basal levels of HER family molecules in all cells or in a subpopulation of cells could prevent the dimerization necessary for signalling and hence tumour growth to occur. This could occur in at least two non-mutually exclusive ways, one in which blocking of HER2 homo-dimerization occurs and another in which blocking of HER2 and HER3 hetero-dimerization occurs.

Trastuzumab has shown significant clinical activity, both as a single agent and in combination with chemotherapy. The treatment is generally well tolerated, with the main adverse effect being cardiotoxicity when used in combination with anthracycline [398]. One clinical trial investigating single-agent trastuzumab as a first line therapy in HER2-overexpressing metastatic breast cancer in 114 patients documented seven complete and 23 partial responses (objective response rate of 26%) [393]. In this study only those patients with a HER2 3+ overexpressing tumour were found to demonstrate responses. One problem of trastuzumab therapy is selection of patients who are likely to respond to therapy. The optimal classification strategy for HER2 expression has been debated [420]. Selection criteria often use immunohistochemical (IHC) techniques or fluorescent in situ hybridization (FISH) to determine HER2 gene amplification levels. Recently, the benefit of trastuzumab treatment was assessed across different subsets of scores for HER2 status defined by FISH or IHC (FISH+, FISH-, IHC 3+, IHC- (0-2+), FISH- IHC- (0-2+)) [421]. Trastuzumab treatment was found to be beneficial for each subset, indicating the possibility that current definitions of HER2 over-amplification might not accurately define HER2 positive tumours, and/or that trastuzumab treatment may be beneficial to a larger range of HER2+/− phenotypes. In another study, 10% of HER2- patients and 48% of HER2+ patients received a clinical benefit (complete, partial, or minor response or stable disease > 6 months) following treatment, indicating the benefit of trastuzumab monotherapy for some HER2-negative patients [393]. This also highlights the facts that not all HER2-overexpressed patients respond to monoclonal trastuzumab therapy. In this model, blocking of HER2 dimerization by trastuzumab treatment might be beneficial as even basal levels of HER2 blocking might be beneficial, and HER2 status definition might not be clinically correct or relevant – i.e. a greater range of HER2 expressions might find clinical benefit from treatment, especially potentially in the case of metastatic disease, as outlined below.

An alternative method of HER family receptor activation involves the binding of the ligand neuregulins (also known as heregulins), cell surface ligands that can also be released as soluble factors [422, 423]. Evidence indicates that neuregulins may play a role in breast cancer and
promote metastatic disease [424], and have been found to promote brain metastases in lung cancer as well [425]. Importantly, expression of transmembrane neuregulin on breast cancer cells is sufficient to cause activation of HER2 on breast cancer cells without HER2 over-amplification and is susceptible to trastuzumab therapy [426]. Clinically, neuregulin transmembrane expression has been found on breast cancer tumours, and is associated with an objective clinical response to trastuzumab therapy even in the absence of HER2 amplification [411]. Neuregulin 1, the ligand for HER3, is found abundantly expressed in the brain [427]. Following binding by neuregulin, HER3 requires dimerization of other HER family receptors for activation, however, only basal levels of expression of other HER family receptors is sufficient for this process [428]. These findings have led Leonard da Silva and Sunil Lakhani (UQCCR) to hypothesise that the neuregulin/HER3 activation axis is an important component for breast cancer colonisation of the brain, and indeed they found HER3 levels to be increased by breast cancer cells residing in the brain (Leonard da Silva and Sunil Lakhani, 2010, in submission). We hypothesise that the downregulation of HER2 by trastuzumab may, in addition to its role in inhibiting HER2 homodimerization, inhibit the neuregulin/HER3/HER2 signalling axis present in breast cancer brain metastases, and prevent growth of tumours (Figure 8.4).

### 8.4 Conclusions

We have shown that tumoursphere-derived cells from one cell line can take to growth in the brain of immunocompromised mice. Future work might benefit from looking at the role that breast cancer stem cells (perhaps tumoursphere-derived cells) play in metastatic disease and particularly assessing new ways to target breast cancer stem cells. Additionally, future work could look into the serial passage of these cells either in the brain directly or through approaches in which cells are injected into the blood supply and analysed as to where they metastasise upon serial transplantation – i.e. lung, bone or brain. If it is to be believed that breast cancer stem cells are largely responsible for metastatic disease, then an experiment of this sort might indicate the propensity for different cancer stem cells of a solid tumour to colonise different distant environments. We have investigated the role that HER family receptors play as one part of the “seed” that allows breast cancer cells to colonise the brain and propose that factors produced by the brain could be part of the “soil” microenvironment that permits this colonisation to take place. Our results indicate that HER2 negative breast cancers that metastasise to the brain might be capable of being treated with trastuzumab. Future work will be needed to assess the utility of treating HER2-negative/low tumours with trastuzumab, or more likely combinations of drugs

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such as trastuzumab, chemotherapy taxanes, and lapatinib. Because of the limited numbers of animals and cell lines tested in our study, this work must be considered exploratory. However, these results open up new avenues for future research into treatment of HER2 non-amplified tumours with therapies aimed at preventing HER family dimerization. Importantly, this work identifies that blocking basal levels of HER2 expression may be beneficial in preventing tumour growth, opening up new therapy options for patients that are HER2 non-overexpressed.
Chapter Nine: Discussion

9.1 Breast Cancer Stem Cells, a Contentious Issue

The field of breast cancer biology, and indeed tumour biology as a whole, has recently encountered a paradigm shifting idea. The concept of tumour evolution as put forth by the clonal evolution theory, in which tumours originate from mutations occurring in one or a few cells leading to uncontrolled growth and proliferation of all cells of the tumour, has been challenged by the concept of the cancer stem cell hypothesis. The cancer stem cell hypothesis, as outlined in this thesis and elsewhere, proposes that tumours are originated from and supported by rare self-renewing cells that have some of the features of somatic stem cells, while the bulk of the tumour is composed of differentiated cells that do not have a tumourigenic or self-renewing capability. A plethora of breast cancer studies previously discussed and cited in this thesis point to the presence of a small subpopulation of cells that display the properties of self-renewal (demonstrated by the enriched capacity of these cells to form tumourspheres) and differentiation found in stem cells, are refractory to chemotherapy and radiation therapy, and may be responsible for tumour initiation and maintenance. While this evidence continues to gather, these cells have not yet been definitively identified and definitive proof of their existence is still unconfirmed. The concept of breast cancer stem cells, and cancer stem cells in general, has misconceptions in terms of nomenclature and concepts. Furthermore, reputable evidence against the cancer stem cell hypothesis has been put forth (commented briefly upon in [429]). This means then that the field of breast cancer stem cells, which is relatively young as the seminal papers came out in 2003-2005, is still a contentious field.

Nomenclature is one of the problems the field is facing. One problem is what to call the cells responsible for the characteristics described in the cancer stem cell hypothesis. Some researchers prefer “cancer stem cells” and others “tumour-initiating cells” or “tumour-propagating cells.” The cancer stem cell hypothesis is based on similarities observed between normal stem cell behaviour and the behaviour of some populations of cells in a tumour. As a result of this, the term “cancer stem cell” seems a reasonable descriptive name for the cells being studied. This name does not necessarily mean that normal tissue stem cells are the target for malignant transformation, which might be more accurately called “the stem cell hypothesis for tumour origin.” It implies instead that since these cells have stem cell-like characteristics they could reasonably be called cancer
stem cells. In some cases it may be true that normal stem cells are the target for tumour transformation; however, it is evident that differentiated cells could also be the target [430]. Additionally, it is possible that somatic stem cells are not the targets of transformation but potentially progenitor cells as has been suggested by similarities between different progenitor cells of breast lineages and different cancer subtypes [39]. In many ways the cancer stem cell hypothesis does not directly try to describe the cell of tumour origin (however, this is no doubt an important component of cancer stem cell research). Instead the cancer stem cell hypothesis describes the way that cancers maintain their formation. As such the term “tumour-initiating cell” has been used to try to clarify the issue by using the word “stem cell” which might imply a direct link to somatic stem cells and to emphasise that these are the rare cells that can initiate a tumour (testable for instance using xenograft assays). Similarly, the term “tumour-propagating cell” has been used to describe these cells. This might all seem like semantics but it does speak to the issues of (i) what do these cells do - they function in many ways like stem cells through such characteristics as self-renewal and differentiation amongst others; and (ii) where do these cells come from - they could be stem cells, progenitor cells, or differentiated cells more work will be needed to rigorously test this and it is possible that no one answer will be correct. As such it is sensible to be clear about these names; however, both names are describing the same set of cells, and importantly these cells are intrinsically different to more differentiated daughter cells (they might for instance be more resistant to treatments or have a greater ability to regenerate a new tumour sharing the same characteristics as the original one). We have made use of the terms cancer stem cells and tumour-initiating cells throughout this thesis.

Another criticism raised against the concept of cancer stem cells has come from work that questions the concept that rare cancer cells are behind tumour growth [431]. Kelly et al. demonstrated that certain malignancies can be sustained by a large proportion of cells that make up the malignancy, calling into question the concept that cancer stem cells are behind all tumours. They investigated the frequency of cells that can sustain tumour growth using mouse tumour cells (pre-B/B lymphoma) injected into non-irradiated histocompatible recipient mice and found that as few as 10 cells from different phenotypic populations of cells could form tumours. It should be noted that their study involved looking at mouse leukaemias and lymphomas, which might be rather more homogenous compared to breast cancer. Work by Kelly et al. also raised several important points about tumour biology and the way that cancer stem cells are assessed in xenograft assays. The foremost issue that they raise is that putative cancer stem cell subpopulations from human tumours are tested for tumorigenicity in mouse models – typically
sublethally irradiated NOD/SCID mice. They therefore argue that studies that have shown that only a minute subpopulation of cells from a human AML can seed a new tumour in NOD/SCID mice reflects not the intrinsic tumorigenicity of that cellular phenotype compared to others in the tumour, but rather the selection for cells that can grow in the foreign microenvironment. Hence, they suggest that xenograft assays of this sort underestimate the proportion of cells that are capable of forming a tumour. They rightly point out that the mouse microenvironment will be important for selecting which cells will grow. Soluble and membrane-bound factors (many of which will not interact with their human cognate receptor) produced within the mouse microenvironment will interact with the injected cells and select for cells that have the best ability to grow inside mice – not necessarily the most tumourigenic cells. However, the importance of using mouse models for studies investigating cancer stem cells should not be understated. The defining property of cancer stem cells or TICs is to form a new tumour, presumably at a concentration less than that needed to form a new tumour from some other set of cells within the original tumour. In order to investigate this, mouse models are needed. One way to reduce the problems discussed here is to humanise aspects of the mouse microenvironment. One way that we and others have looked at this is to humanise the fat pad of mice using a mixture of irradiated and non-irradiated human fibroblasts cells placed into the cleared fat pad of mice before injection of tumour cells. Xenograft models such as this will be more capable of sustaining the growth of human cells and might be better for assessing the frequency of cells that are capable of tumour initiation.

Another critical appraisal of the field of cancer stem cells came from Kern and Shibata arguing that from a review of the literature, the mathematical evidence for the existence of cancer stem cells is weak [432]. Their critique of the field raises many interesting questions related to numerical discrepancies that exist in isolating, identifying and targeting solid tumour stem cells. They point out that methods used for isolating cancer stem cells often are contaminated with non-stem cell cells or that the opposite occurs, that cancer stem cells are still present in the non-stem cell population. As such these studies are not utilising a true population of cancer stem cells compared to a true population of non-stem cells. This is a problem faced by many investigators including us, and one that will need to be answered. Our use of the tumourspheres assay to enrich for cancer stem cells does not necessarily isolate them from other cells. We have demonstrated in our work the use of a mathematical model to assess LTP cell symmetric division rate and have shown that this is a useful exercise for predicting tumorigenicity in mouse models. While this mathematical model does not irrefutably prove the existence of cancer stem cells, its use does
answer the challenge of Kern and Shibata to use mathematical models as a guide to interpret cancer stem cell studies.

Clearly the field of cancer stem cells is a contentious one with many gaps in understanding still waiting to be resolved. We have made use of the \textit{in vitro} tumoursphere assay to investigate the functional and phenotypic properties of cells composing tumourspheres and how they relate to characteristics of cancer stem cells and \textit{in vivo} xenograft assays to further characterise tumoursphere tumorigenicity. We have utilised both breast cancer cell lines and primary and metastatic breast tissue to conduct our analysis of tumoursphere growth. The use of breast cancer cell lines and primary tissue samples both have pros and cons associated with them (as reviewed in [152]). Notably, cell lines are useful in that they demonstrate sustained, reproducible growth in tissue culture and can be selected to investigate the influence of subtype on tumoursphere formation. But their use suffers from the fact that most cell lines originate from pleural effusion samples and hence do not represent the primary original tumour tissue and worse the potential to use false lines. Furthermore, these cells have been already selected for their property to be able to grow \textit{in vitro}, hence skewing the analysis away from the \textit{in vivo} components of the tumour. Primary tissue is of course a preferable route for investigation, but we and others have found difficulty generating cell lines from primary tissue. Overall, the use of the tumourspheres model in conjunction with xenograft assays should form an integral part of studies on breast cancer stem cells. The use of these models could even find use in clinical trials as a way to assess the ability of inhibitors to target cancer stem cell self-renewal (as has been described in [119]).

Characterising TICs and the determination of factors involved in forming TICs will have an important impact on cancer therapies. Current treatments of cancer have shown efficacy in removing the bulk of differentiated cancer cells, while failing to eliminate the TICs responsible for tumour relapse. Additionally, it will be important to target those cells that are capable of metastasising to form new tumours before the dissemination occurs. Common mechanisms involved in stem cell self-renewal and cancer initiation are starting to be uncovered. The challenge now will be to find ways to eliminate those rare TICs by targeting the common growth signalling pathways, while sparing normal stem cells or by targeting molecules that are differentially expressed by cancer stem cells compared to differentiated cells within a tumour. Understanding these differences between tumour stem cells and differentiated cells will go a long way towards developing a rational approach to eliminate the rare cells responsible for tumour
initiation, maintenance and relapse. This has been the focus of the work presented within this thesis.

9.2 Importance of this thesis work in the context of the field of breast cancer stem cells

Discussion of results is presented in their individual chapters. What follows is a general overview of the importance of this work. The work presented in this thesis is focused on validating the use of the tumourspheres assay for assessing the properties of breast cancer stem cells, and characterising tumoursphere functional and phenotypic properties that are different from differentiated/monolayer culture. The tumourspheres assay has been used by several investigators to show self-renewal or a self-maintenance capacity of cells within tumours, often times in conjunction with other assays such as flow sorting for aldeflour enzyme activity or CD44+CD24-phenotype. We investigated the use of the tumoursphere assay as a way to enrich for breast cancer stem cells and to model stem cell growth in vitro and predict behaviour of tumour growth in vivo (Chapters 3 and 4). The use of a mathematical model demonstrating that growth in vitro is correlated with growth in vivo is a novel finding of this work, and goes a long way towards validating the use of the tumoursphere system for characterising breast cancer stem cells. The second focus of this thesis is to understand the differences between putative breast cancer stem cells and the more differentiated cells that compose the bulk of tumours. This is an important component of breast cancer stem cell research. Breast cancer stem cells are thought to be more resistant to conventional treatments such as radiation and chemotherapy than differentiated cells. Understanding the reasons for these functional and phenotypic characteristics is vitally important. Equally as important is to identify markers that can be used to isolate or target breast cancer stem cells. This thesis has investigated the effects radiation has on sphere formation (Chapter 7) and has looked into the phenotypic and morphological properties of tumourspheres to identify potential markers of BCSCs (Chapters 5 and 6). Phenotypic properties of spheres were assessed using flow cytometry, immunohistochemistry, and proteome analysis. Importantly, the work presented here made use of a large panel of breast cancer cell lines representing different subtypes of breast cancer – a strategy that has not be well used in the literature. Identification of potential markers of BCSCs has led us to investigate the effects of inhibitors of those markers (specifically trastuzumab for HER2 expression and LacNAc for galectin-3 expression). The identification of HER2 as a potential marker of breast cancer stem cells led us to investigate the use of trastuzumab treatment to target cells that express HER2 (but not clinically overexpress
HER2) in a mouse brain metastases model for both tumourspheres and adherent cells (Chapter 8). This project presents intriguing data suggesting that HER2 non-overexpressed cancers can be treated with trastuzumab therapy. Overall, the data presented in this thesis validates the use of the tumourspheres model for assessing BCSCs; progresses the fields’ understanding of how the tumoursphere assay can be used for modelling stem cell behaviour of cancer cells; and demonstrates some relevant findings for markers expressed by BCSCs that could be used for potential therapy.

9.2.1 Discussion of data from chapter three. The culture of tumourspheres from primary breast cancer patient samples is not widely reported on in the literature. We have demonstrated the capability of some samples to culture as tumourspheres, albeit for a short time. None of the primary samples tested were capable of growth beyond two or three passages as spheres, or indeed as adherent cultures. The fact that no long term cultures were established could indicate that sphere formation from these lines was driven by short term proliferating cells and not long term proliferating cancer stem cells. A breast cancer brain metastasis sample that was also cultured in tumoursphere-promoting conditions was found to be capable of longer self-renewal. This could be indicative of metastatic samples being enriched in cells capable of forming tumourspheres, which is indeed what has been shown from studies that rely heavily on pleural effusion samples compared to primary breast tumour. Alternatively, this could indicate that the culture conditions are biased towards culturing cells that have previously been growing in the brain microenvironment. This later idea is possible given the fact that the tumoursphere culture system was originally set up to culture neural stem cells. This part of the project also validated the use of a mouse model (specifically the humanised fat pad technique in immunocompromised mice) as an “in vivo” incubator to allow for the growth of primary human tumour cells for later culture as tumourspheres. This might be a necessary requirement to generate larger number of cells to work with as well as for potentially enriching for tumourigenic cells. Thus, this system might be useful for culturing all patient tumour samples in a mouse before establishing culture in tissue culture. Overall, this part of the thesis validates the potential to grow primary and metastatic breast cancer samples as tumourspheres in vitro and to grow in vivo tumour cells from primary samples that are later capable of growth as tumourspheres in vitro. As a result of only having a few samples capable of culturing for only a few passages as tumourspheres, we moved on to testing the ability of cell lines for culture in tumoursphere-promoting conditions.
9.2.2 Discussion of data from chapter four. At the current time stem cells and cancer stem cells are difficult to phenotypically describe. There exist few unique sets of markers that can be reliably used to isolate and discriminate them against non-stem cells or differentiated cells. Therefore, we have endeavoured to functionally describe stem cells using the tumoursphere assay. This assay allows the demonstration of self-renewal upon serial passaging and has been cited and discussed in this thesis. Generally, this assay makes use of calculating the sphere forming efficiency of cells over serial passage. We have made use of a mathematical model to assess fold expansion rate of cells grown as tumourspheres, the number of long-term proliferating cells within a tumoursphere, and the symmetric division rate of long-term proliferating cells that does not require calculation of the sphere forming efficiency but rather assessing the expansion of the cells in tissue culture. We found that both SFE and fold-expansion rate were relatively stable over serial passage of cells for several cell lines tested. This could indicate that LTP cells are driving tumoursphere formation and maintenance. However, we found that there was no correlation between SFE and either fold-expansion or LTP cell numbers. SFE was found to overestimate LTP cell presence for cell lines and this validates work by others finding a similar trend for neural stem cells [168]. SFE might be an overestimate of stem cell frequency because spheres can form in part from early progenitor cells or clumping in addition to forming from LTP cells. Hence, modelling LTP cell numbers and division rates is more accurate using the mathematical modelling technique described in this thesis. Interestingly, we found that growth as adherent cells and growth as tumourspheres was correlated. This might indicate that the growth of cells in both culture conditions is sustained by the same population of cells – LTP cells. This is the first time to our knowledge that the correlation between growth of adherent and tumoursphere cells has been reported for breast cancer cell lines. Overall, our findings from this part of the thesis suggest that while SFE can be a useful assay to show self-renewal of a population of cells comprising spheres, the determination of fold-expansion rate over serial passages might be a better way to enumerate stem cell numbers. This concept was further validated when we showed that symmetric division rates for cell lines determined in tumoursphere-promoting conditions could be used to predict tumour growth in mouse models. Application of these findings could have broad impacts on the field of breast cancer research allowing an in vitro assay for testing the effects of therapies aimed at stem cells or through testing populations of cells expressing candidate stem cell markers for their presence of LTPs.

9.2.3 Discussion of data from chapters five and six. We next wanted to examine the ability of different subtypes of breast cancer to form spheres with the aim to examine them
phenotypically to determine putative breast cancer stem cell markers. Many (but not all) of the breast cancer stem cell studies in the literature use only a small panel of cell lines and often do not have lines that represent various subtypes of breast cancer. One outcome of subtype specific differences in breast cancer is in identifying different treatment options. In the case of breast cancer stem cells, this also means identifying potentially different markers for the different subtypes. As such we wanted to make sure that our study incorporated a panel of cell lines (we used 25) that represented different molecular subtypes – primarily along a basal-like luminal-like division. We found that tumourspheres could be propagated from a variety of different subtypes of cells. No correlation existed between sphere forming capacity and subtype or tumorigenicity. These findings were not surprising giving the concept that breast cancer stem cells should be present in tumours despite subtype and that non-tumourigenic cell lines can also form tumourspheres. Tumourspheres from luminal lines were found to be different in morphology to tumourspheres from basal lines. This is similar to what is seen from adherent cultures in differences between the two. Luminal tumourspheres were cohesive structures often times with lumens or clefts on the inside. Basal tumourspheres on the other hand were non-cohesive structures. We propose that the growth of cell lines as tumourspheres, in addition to being a culture method for enriching BCSCs, is a better model for growing cells than adherent culture as tumourspheres more accurately model the in vivo situation of tumour growth. Assessment of the CD44/CD24 co-staining of cell lines revealed that this phenotype correlates with basal-ness of cell lines and not a BCSC marker per se as has been proposed by other researchers. We further assessed a variety of cell surface markers on tumourspheres and have validated some potential markers of interest such as HER2, EpCAM, CD49f and MUC1. Each of these markers has previously been described by other researchers as being involved in BCSC biology.

In order to identify some new markers of BCSCs we decided to utilise proteomics to compare MCF7 tumourspheres to adherent cells or early passage tumourspheres to late passage ones. This research identified a variety of different molecules differentially expressed between the populations. One marker identified was galectin-3. We further assessed the ability of an inhibitor of galectin-3 to inhibit sphere formation in a variety of cell lines and found that it does inhibit sphere formation in MCF7 cells and HBL-100 cells. Future experiments will be needed to further identify and confirm other proteins differentially expressed between spheres and monolayer cells. Overall, the experiments presented in these parts of the thesis validate the use of previously described cancer stem cell markers and describe some more potential molecules for future validation.
9.2.4 Discussion of data from chapter seven. We also assessed the effects that radiation has on sphere formation and found, as previously described by others [90], that irradiation increases the sphere forming capacity of MCF7 cells. These findings validate the concept that breast cancer stem cells are relatively radioresistant and can increase in numbers following short courses of fractionated irradiation. However, not all cell lines that we tested had this capacity, in fact only MCF7 out of five lines tested had this capacity. This could mean that only MCF7 cells have true cancer stem cells (meaning cancer stem cells that exhibit all of the known functional criteria typically given to cancer stem cells) or that cancer stem cells might not be as radiosensitive as proposed. Recently, others have proposed that CD133+ glioblastoma stem-like cells are radiosensitive [359]; giving credence to the notion that perhaps not all (breast) cancer stem cells are radioresistant. This portion of the thesis also assessed the cell cycle distribution either with or without radiation for adherent cells and tumourspheres and slow-cycling cells within adherent or tumoursphere populations. Adherent cells and tumourspheres demonstrated a dose-dependent increase in the G2 phase of the cell cycle in response to irradiation. The frequency of cells in the G2 phase of the cell cycle was found to be increased in slow cycling cells compared to all cells irrespective of irradiation. These results suggest that adherent cells and tumourspheres respond in similar ways to radiation treatment in terms of changes in cell cycle distribution – the trend in increasing the frequency of cells in G2 phase post-irradiation. Determining other differences in the ways that adherent cells and tumourspheres respond to irradiation will need to be more properly assessed in the future. The finding that slow-cycling cells are enriched in the frequency of cells in G2 phase indicates that this population (a population with some of the characteristics of BCSCs) might be less radiosensitive than the general population, as an extended G2 phase could be used by cells as a mechanism to prolong repair of DNA damage. Further research will be required to investigate this possibility.

9.2.5 Discussion of data from chapter eight. Finally, we have examined the growth of breast cancer cells within the brain microenvironment. We were interested in doing this for several reasons: (i) cancer stem cells may be the cells responsible for brain metastases and as such this research would further our understanding of cancer stem cells; (ii) metastatic breast cancer is an increasingly important disease component of breast cancer; and (iii) our finding of HER2 expression increasing on tumourspheres prompted us to investigate the effects of trastuzumab treatment in an in vivo setting using cell lines that are not clinically defined as HER2 overexpressing. Results from this part of the thesis indicated that tumourspheres from the MCF7
cell line did not grow as quickly in the mouse brain as did adherent cells. This might indicate that cells that comprise tumourspheres are not as tumourigenic as adherent cells. It would be interesting; however, to analyse the serial passage of tumoursphere-derived cells and adherent cells in an intracranial model to assess whether tumoursphere culture enriches for cells capable of establishing tumours over serial passage in this model. Importantly, we have shown that both cells grown as tumourspheres and as adherent cells are capable of establishing tumours in the mouse brain microenvironment and both show response to treatment with trastuzumab. Interestingly, we found that two HER2-negative/low breast cancer cell lines injected into the brain could be treated with trastuzumab therapy to prevent or delay tumour progression. We hypothesise that the downregulation of HER2 by trastuzumab may, in addition to its role in inhibiting HER2 homo-dimerization, inhibit the neuregulin/HER3/HER2 signalling axis present in breast cancer brain metastases, and prevent growth of tumours. This work suggests that clinical practice should treat a broader range of HER2 expressing tumours with treatments against HER2 than is currently done. Future work would be needed to demonstrate these findings in more cell lines.

9.2.6 Overall. Overall, the results presented in this thesis provide compelling evidence for the use of the tumoursphere model for assessing BCSCs. The results demonstrate that the SFE assay is a good way to identify the presence of LTP cells, but that by using the mathematical model described here better enumeration of LTP cell numbers can be accomplished, and that this model has predictive value for determining tumorigenicity in vivo. The results validate the growth of some breast cancer cell lines as tumourspheres, as previously described, and confirms the ability of several more, thus extending the number of breast cancer cell lines known to culture as tumourspheres. Importantly, we have made use of a large panel of breast cancer cell lines representing various clinical subtypes of breast cancer and have demonstrated how basal and luminal tumourspheres are different to each other morphologically. The results presented here also demonstrate several potential markers for identifying breast cancer stem cells. Among these are some that have been previously cited in the literature such as MUC1, HER2, CD49f and EpCAM. We further identify galectin-3 as a potential new marker for BCSCs. There is still a need for identifying more markers expressed on cancer stem cell populations. This is undoubtedly a goal for many researchers and one that will be important for developing treatment for long term remission of breast cancer patients. Clearly more experiments will be needed to further validate the existence and phenotype of breast cancer stem cells.
9.3 Limitations and shortfalls/pitfalls

This work was not without limitations and problems. One major limitation of this research was finding a suitable mouse model for growing primary tumour tissues. Originally SCID/Beige mice housed at AIBN (UQ) were used for some experiments (not presented in this thesis). Then, after establishing techniques at QIMR learned at the Queensland Brain Institute, UQ, NOD/SCID and RAG\(^{-}\) mice were used. Neither of these mouse strains were found to be useful for tumour implantation. Indeed we found evidence for cellular immune components (T-cells, B-cells and NK cells) in some mice (data not presented). As such we had to import and establish a new colony of mice (NOD.Cg.Rag) and this took valuable time away from research. However, the end result was a mouse model that was useful for our purposes.

Another major limitation was that access to patient primary breast cancer samples was sporadic and samples became less frequent and of a lesser size after 2008. Unfortunately, this occurred at the same time as the mouse model was finally being optimised. This meant that fewer samples were tried in our in vivo “incubator” mouse model than could have been. However, enough primary patient samples were obtained to examine our goal objectives of testing sphere formation in vitro and testing the capability to culture tumour chunks in the humanised cleared fat pad of immunocompromised mice for expanding cells for further analysis in tumoursphere-promoting conditions in vitro. The mouse model has also been set up for testing the ability of the tumoursphere mathematic model for predicting tumorigenicity. Now that the mouse model has been optimised, future work in the lab can use this system for testing a variety of questions concerning tumour biology, and this has been a direct result of this thesis work.

Another serious problem associated with any work involving breast cancer cell lines is the possibility of using false cell lines, in other words not the cell line that the researcher believes they are using (reviewed in [152]). This is important for our work as we compared the tumourspheres from basal and luminal lines and hence it is important that we use the correct line to get meaningful results. Obviously, this is also important in that reporting data on a cell line that is not what it is claimed to be will cloud the field with useless information. The widespread problem of false cell lines is illustrated by the finding that out of the 252 new cell lines deposited at the Berman Cell Line Bank, 18\% were found to be cross-contaminants [433, 434]. To combat this problem we sought to characterise the cells used in these experiments and determine if they fit the phenotypic description they have in the reviewed literature. This is important in that it
reassures the researcher that the cells are morphologically and phenotypically similar to what other researchers report, but does not necessarily identify the cell line. Whenever possible we used cell lines bought directly from ATCC. We also used STR profiling as a way to screen cells where we had STR profile information. Unfortunately not all of the cell lines that we used had readily available STR profiles for comparison. Results of the STR profiles that we conducted revealed that the BT-474 cells and the MDA-MB-157 cells that we used for some experiments were not in fact those cell lines. As a consequence, results with those cell lines were discarded and, in the case of BT-474, new cells were bought from ATCC. Similarly, a lot of early research done for this thesis used the MDA-MB-435 cell line. After the start of this work it was reported that MDA-MB-435 is likely a melanoma cell line [172]. As a result, much of the MDA-MB-435 data was not used (however, we still report in chapters 4 and 5 the ability of this cell line to form tumourspheres). Clearly, the problem of contamination with false cell lines is an important one that affects many researchers; however, we have strived to eliminate this variable from our work. A slightly related problem is the possibility of contamination of cell lines with mycoplasma infections, which can invalidate the results obtained. We have conducted regular mycoplasma testing throughout the course of this work, and have strived to use data from mycoplasma free cell lines. We have had mycoplasma positive tests following screening and have attempted to either clear the mycoplasma infections or use different stocks of clean cell lines.

Tumourspheres can be generated using a variety of different culture methods and mitogens. We have made use of one culture method that is popularly used for growing neural stem cells and cancer stem cells from glioblastoma (amongst other cancers). Methods for growing spheres are fairly similar but could have some crucial differences. They are mostly serum-free systems composed of DMEM/F12 or some other suitable medium that contain similar amounts of BSA, bFGF, and EGF. Some systems also use hydrocortisone, heparin, or insulin. However, one major way that culture conditions differ among various reports is in the source of other proliferation supplements added. Some reports use B27 (Sigma) as a source of stimulation [78, 359]; others use proliferation supplements from Stem Cell Technologies such as Neurocult™ [168]; still other reports do not specify the use of proliferation supplements to grow spheres [90]. The exact way that these proliferation supplements work to stimulate sphere formation is not entirely known. We have made use of Neurocult™ throughout these experiments. After the start of these experiments, Mammocult™ was introduced by Stem Cell Technologies. This product is supposed to be better for growing mammospheres/tumourspheres than Neurocult™. This is presumably because mammary specific mitogens are present in the mixture. Clearly, various ways exist to culture
spheres, with the possibility that one culture method might work for one cell line, while another culture method does not. In fact we have seen this in our own work, where human Neurocult™ was needed for the growth of BT-20, while mouse Neurocult™ did not work. To better define what cell lines are capable of forming spheres perhaps other culture methods should be used (i.e. test each cell line with B27 or Mammocult™ containing media). This was not done because spheres were obtained using Neurocult™ for a number of cell lines, and also because there was a lack of time to follow up on this idea as it was not considered as important as testing our goal hypotheses.

9.4 Avenues of research to develop from this thesis

The results presented in this thesis certainly answer some questions about the nature of tumourspheres and BCSCs. Additionally, a variety of future experiments could be performed based on these results. Among them would be to; (i) assess tumorigenicity of the markers we have identified either alone or in combination with other markers; (ii) test inhibitors of markers we have identified to block cancer stem cell self-renewal and tumorigenicity in vitro and in vivo; (iii) characterise the response of tumourspheres to combination radiotherapy/chemotherapy; (iv) test the use of immunotherapy against BCSCs as a potential approach to eliminate minimal residual disease; (v) conduct a more thorough proteome screening to assess more markers of breast cancer stem cells; and (vi) determine the metastatic capacity of BCSCs. Following is a brief description of some future experiments that the results from this thesis suggest.

9.4.1 Tumorigenicity. One set of experiments would be to assess the tumorigenicity of cells expressing the various markers we have identified (MUC1, CD49f, HER2, EpCAM, Galectin-3) alone and in combination with other markers (aldeflour activity for instance) in mouse models. Towards this aim, we have tested the ability of our tumourspheres to express aldeflour (data not shown). A variety of the cell lines grown as tumourspheres do express aldeflour activity. Future experiments could look at the co-expression of this enzyme with the putative markers of BCSCs that we have defined. Then after sorting for a population of interest, the next set of experiments would be to see if SFE is different between the two populations, possibly identifying a population that is enriched in cancer stem cells (also other in vitro experiments would need to be conducted such as long term serial passage and mathematical modelling of LTP symmetric division). Finally, assessing tumorigenicity of the different
populations in a mouse model would be necessary to see if one population is truly enriched in tumourigenic capacity. Importantly, it would be ideal to show that the tumour produced \textit{in vivo} from a particular subpopulation of cells is capable of generating a heterogeneous tumour and that further serial passaging of that particular subpopulation of cells is possible in additional mice (perhaps in contrast to the other non-cancer-stem cell population). Other related experiments would be to assess the ability of tumour cells with knocked down expression (perhaps using siRNA) or enhanced expression (perhaps using a lentiviral system) of the putative BCSC markers in the same \textit{in vitro} and \textit{in vivo} systems for modelling cancer stem cell growth. Experiments of this type are needed to show the validity of the putative cancer stem cell markers we have identified.

\textbf{9.4.2 Inhibitors of BCSCs.} Another set of future experiments would be to assess the ability of inhibitors of galectin-3 to block tumour formation in mouse models. We have shown \textit{ex vivo} that galectin-3 is expressed on putative breast cancer stem cells from MCF7 cells, and that inhibition of galectin-3 results in a dose-dependent decrease in sphere formation. Future experiments should be conducted to assess these effects on TICs \textit{in vivo}. This work could use LacNAc or perhaps another inhibitor of galectin-3 that might be more appropriate for \textit{in vivo} use. Further testing of inhibitors of HER2 should also be done. We have shown \textit{ex vivo} that trastuzumab therapy does not block sphere formation (and hence self-renewal); however, the ability of inhibitors of HER2 to affect TICs \textit{in vivo} has not been fully assessed. Future experiments should be conducted to assess the ability of trastuzumab or other HER2/EGFR inhibitors to block tumorigenicity of BCSCs \textit{in vivo}. Other molecules such as mitocans, anticancer molecules that cause apoptosis by destabilising mitochondria, could also be assessed [435]. Indeed, the work described in this thesis outlines a rational approach to test the ability of a variety of therapies to target BCSCs with the readout being sphere formation or LTP symmetric division \textit{ex vivo} and tumorigenicity in mouse xenografts. Hence, a variety of therapies could be readily tested in a fairly high-throughput fashion to see which therapy has an effect on blocking cancer stem cell self-renewal.

\textbf{9.4.5 Characterise the response of breast tumourspheres to radiotherapy and combination radiotherapy/chemotherapy.} Results from this thesis suggest that some breast cancer cell lines are capable of surviving radiotherapy and that breast cancer stem cells play a role in this. Recently, the importance of targeting multiple
pathways of DNA double-strand break repair has been realised for achieving effective treatment of breast cancer (reviewed in [436]). The radiation response and the response to combinational therapy of various cell lines (luminal and basal-like) grown under monolayer, tumoursphere-promoting and 3D culture systems could be compared to each other. Additionally, the comparison of different subpopulations (enrichment for putative breast cancer stem cells) could also be assessed. Combinations of therapies could include combinations of the use of traditional chemotherapy such as doxorubicin and docetaxel, high dose and low dose radiation, targeted radionucleotide treatment, inhibitors of the checkpoint kinases Chk1 and Chk2, and inhibitors of DNA repair pathways. This assessment could consist of:

1) Analysis of changes in the frequency of various cell surface stem cell markers post-treatment.

2) Clonogenic survival of the various culture conditions and subtypes.

3) Sphere forming efficiency (a measure of self-renewal activity) could be assessed post-treatment for the various culture conditions and sorted populations to test the possible enrichment of BCSCs post-treatment. In conjunction with this, assessment of long term proliferation and rate of symmetric division could be conducted, as well as tumorigenicity studies in immunocompromised mouse models.

4) Analysis of changes in expression/activation of signalling pathways such as Notch-1/4 (amongst others) that have a role in self-renewal.

5) Analysis of γH2AX clearance post-treatment could be assessed for the various conditions and subtypes.

6) Analysis of markers of cell death such as active-caspase-3 and annexin-V expression post-treatment.

7) Analysis of the expression of free radicals post-treatment could be conducted for the various samples to see which culture condition/cell phenotypes are most sensitive to irradiation and combinatorial therapy.

8) Analysis of the various samples’ cell cycle distribution before and after treatment could be conducted as well as expression of various cell cycle check point regulatory proteins (Chk1 and Chk2). In particular, assessment of the various samples for frequency of cells in G2 phase of the cell cycle could be conducted. If BCSCs are found to be delayed in G2 phase (as our results and the results of others suggest), then inhibitors of cell cycle checkpoints might be very interesting to explore in combination with radiotherapy to eliminate BCSCs.
9) Determination of the tumorigenicity and ability to repopulate a tumour for the various cell culture conditions/isolated cells that survive these novel combinatorial treatments in xenograft assays.

### 9.4.4 Immunotherapy targeted against BCSCs.

One therapy that we have proposed for being useful for targeting cancer stem cells is immunotherapy [108]. More recent advances in the use of immunotherapy to target cancer stem cells has shown promise in models of colon, ovarian, glioma and breast cancer and using a variety of immunotherapy strategies including NK cells, γδT-cells, and CTL (reviewed in [437]). Recently, a report has shown the promise of immunotherapy for targeting tumour cells with some of the characteristics of cancer stem cells in breast cancer models utilising MCF7 cells [438]. We have identified some proteins that have enhanced expression on tumourspheres compared to adherent cells. Targeting of these proteins, or others identified in other approaches, might eliminate the cancer stem cell population and hence the ability of a tumour to maintain itself. We propose the use of dendritic cell immunotherapy to direct a CTL immune response towards known TAAs expressed by BCSCs using either peptides, DNA, RNA, or viral vectors to insert the antigens of interest. Another approach would be to utilise DCs that have been exposed to whole tumour lysates from the BCSC pool so that multiple TAAs could be presented without the need for identification. It would be ideal to show that this approach would work in cancer cell lines, as well as with patient tumours using autologous dendritic cell preparations. At present, this might be difficult to achieve due to the need for obtaining ethics approval and patient blood, as well as optimising growth of patient tumour samples. However, if this could be achieved it would be an interesting and novel finding bridging the fields of cancer stem cell biology and immunotherapy, as well as having an impact on clinical care. This approach might also involve better characterising the expression of cancer testis antigens on cancer stem cells, as these antigens are immunogenic and would be ideal targets for immunotherapy if it could be proved that they are expressed by cancer stem cells.

### 9.4.5 Proteomics.

Another set of experiments would be to compare the proteome expression of a variety of tumourspheres, or subpopulations of cells in tumourspheres to each other, to see if a cancer stem cell proteome signature could be found, identifying novel pathways or markers expressed by cancer stem cells. This might be especially useful if all membrane proteins could be isolated and compared, as this information would allow for better isolation of cancer stem cells in the future (via FACS for instance). Additionally, this could be expanded to include comparing the proteome of basal-like tumourspheres to luminal-like tumourspheres, or to comparing the
proteome of different subtypes of breast cancer to different normal progenitor cells in the breast. This might uncover connections between the cell of origin of tumours and the subtype, similar to the gene expression profiling data suggesting a link between different normal human epithelial and stromal subsets and different breast cancer subtypes [39]. Proteomics research offers many possibilities for identifying signalling pathways and other proteins differentially expressed between two populations of cells. Certainly in the future proteomics will be better used for assessing BCSCs and could lead to new breakthroughs in targeting these cells.

9.4.6 Metastatic disease and BCSCs. There is a real risk of metastatic disease following breast cancer diagnosis, and much remains to be understood about the processes regulating metastatic disease. Research has hinted at a link between metastatic disease and breast cancer stem cells, showing that pleural effusion samples (metastatic disease) can be cultured as tumourspheres, and that this enriches for tumourigenic cells [82]. Other studies using breast cancer cell lines have shown that IL-8 can stimulate aldeflour expression and increase tumoursphere formation [88]. Charafe-Jauffret et al. suggests that IL-8, which has a previously reported role in tumour metastasis, promotes the invasion and chemotaxis of cancer stem cells. Thus, cancer stem cells may be responsible for tumour metastasis. As such, we have endeavoured to explore the growth of breast cancer cell lines in mouse brains. Our work, primarily focused on investigating the use of trastuzumab to treat HER2 negative/non-overexpressed breast cancer cells, revealed some intriguing results. These results should certainly be further explored, both in experiments using more mice (particularly for MDA-MB-231 cell line) and more controls (such as using rituximab and different amounts of trastuzumab treatment), as well as in vitro to determine how trastuzumab treatment affects tumour cells that do not express HER2. However, this work also demonstrated that cells derived from MCF7 tumourspheres did not grow as quickly in the mouse brain as did adherent cells. It would be instructive to conduct additional tests to determine whether tumoursphere culture enriches for cells capable of establishing tumours over serial passage in this model. This might also entail testing if different putative BCSC subpopulations can initiate a tumour in the mouse brain and be serially transferred.

Additionally, it would be ideal to establish a model to test metastatic disease of two populations via injection of cells into the carotid artery or the left cardiac ventricle as described by Steeg et al. [390]. It is possible to enrich for cells that have a metastatic potential to a particular organ by taking metastatic cells from that organ and retransplanting them back into the mouse. Over serial passage there is an increase in cells going to that particular metastatic site. It would be
particularly interesting if those cells were enriched in the ability to form tumourspheres or culture as tumourspheres. That might be a novel method for assessing the ability of BCSCs to initiate and maintain metastatic disease. Testing the ability of BCSCs to initiate metastatic disease could be an important avenue for future research and could have an impact on the therapy of breast cancer.

Another important aspect of metastatic disease for epithelial malignancies, which we did not formally explore during this thesis, is the involvement of cells undergoing an epithelial-mesenchymal transition (EMT). This phenomenon involves epithelial cells losing expression of E-cadherin and acquiring the migratory phenotype of mesenchymal cells; and hence, acquiring the ability to travel to metastatic sites without being affected by traditional therapies [439-441]. Once at the site of metastatic disease the cells undergo a mesenchymal-epithelial transition. The EMT is thought to be influenced by a number of signals in the neoplastic microenvironment, including activation via EGF and FGF (both molecules that are used in the tumoursphere-promoting culture conditions). A link has now been suggested between cancer stem cells and the EMT [442, 443]. Aktas et al. have demonstrated that the stem cell marker ALDH1 and a variety of EMT markers are overexpressed in circulating tumour cells of metastatic breast cancer patients [442]. It would be instructive to characterise these cells for the functional attributes of BCSCs. One future experiment that could be done would be to characterise the expression of EMT markers (Twist, Akt2, Pi3k and vimentin amongst others) in tumourspheres compared to adherent cells in our panel of cell lines. It might be additionally instructive to do this comparison between luminal tumourspheres and basal-A/B tumourspheres to determine which groups are capable of undergoing an EMT. Our current results demonstrate that luminal lines lack expression of vimentin, while basal lines express vimentin (chapter five). Perhaps as an initial experiment only basal lines would be compared between adherent cells and tumourspheres for changes in expression of EMT markers. It would also be interesting to assess the EMT phenotype in cells that did not form tumourspheres in tumoursphere-promoting culture. A number of cell lines that we have tested were unable to form spheres, but that does not necessarily mean that they are not expressing some markers of EMT and potentially BCSCs. It would be interesting to further characterise these cells for markers of EMT in tumoursphere-promoting conditions.

9.5 Conclusions

The ultimate goal of this research is to identify and characterise the cells that are responsible for breast cancer tumour initiation, maintenance, metastasis, treatment resistance and tumour relapse.
Breast cancer is the leading cause of cancer related deaths for women in the Western world. At the present time, women in Australia have a one in ten lifetime chance of developing breast cancer, with women in the United States of America having a one in eight chance [444]. Identifying the ways that breast cancer stem cells are different to differentiated cells will go a long way to developing novel rational treatment strategies targeting these cells. Currently the Response Evaluation Criteria in Solid Tumours (RECIST) is used for assessing the effectiveness of new therapies in clinical trials, with the primary endpoint assessed in most trials being tumour shrinkage. However, this does not always correlate with patient survival, as cancer stem cells can lead to tumour relapse. New techniques need to be used that take into account the cancer stem cell compartment of tumours and that can be used to test the ability of drugs to target this compartment. The work presented here demonstrates that the tumoursphere assay is a useful model for studying breast cancer stem cells. This model is ideal for monitoring the number of LTP cells in tumours and could help in monitoring the effectiveness of various drugs to target cancer stem cells in new clinical trials. We propose that in the clinical setting, combination therapy of bulk tumour targeting and cancer stem cell targeting will be needed to lead to clinically meaningful remissions of breast cancer. Future studies looking at immunotherapy against breast cancer stem cells could accomplish this goal. These studies could make use of the knowledge developed in this thesis and hopefully will lead to improvements in monitoring disease, treatment options and quality of life for breast cancer patients.
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