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THE ROLE OF SPY1 IN BREAST CANCER STEM/PROGENITOR POPULATIONS: IMPLICATIONS FOR BREAST CANCER TREATMENT

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by
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ABSTRACT

Breast tumours are heterogeneous and contain populations of cells with stem-like qualities that are characterized by long term self-renewal capability and the ability to generate more differentiated progeny. This model for carcinogenesis carries significant clinical implications as cancer stem-like cells have enhanced protective mechanisms that make them resistant to conventional therapies. Designing treatment options to target this aggressive population requires an understanding of the mechanisms regulating their growth and fate decisions, including cell cycle regulation. The protein Spy1 is an atypical cyclin that enhances cell proliferation and overrides senescent barriers. Spy1 has demonstrated roles in maintaining stemness in the brain and is elevated in human breast carcinoma. This study demonstrated that Spy1 is a driver in the population of stem-like cells across a number of different breast cancer cell lines. The findings in this study may have clinical implications toward targeted approaches in the treatment of breast cancer.

DEDICATION

This thesis is dedicated to the loving memory of Flavia Carlini. May your beautiful spirit live on through your daughters Kaitlin and Karissa and be a constant reminder of the preciousness of life.

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LIST OF ABBREVIATIONS

MaSC	mammary stem cell
MMTV	mouse mammary tumour virus
Lin^-	negative for lineage markers
CD	cluster of differentiation
ADAM	a disintegrin and metalloproteinase
CBF	C promoter binding factor
Cdk	cyclin-dependent kinase
CAK	cyclin-dependent kinase -activating enzyme
CKI	cyclin-dependent kinase inhibitor proteins
ATP	adenosine triphosphate
RINGO	Rapid Inducer of G_2-M progression in Oocytes
Spy1	SpeedyA1
BTIC	brain tumour-initiating cells
HER2	human epidermal growth factor receptor 2
ER	estrogen receptor
PR	progesterone receptor
CSC	cancer stem cell
SCID	severe combined immunodeficient
TGF-beta	transforming growth factor beta
ALDH	aldehyde dehydrogenase
BODIPY	boron-dipyrromethene
BAAA	boron-dipyrromethene aminoacetaldehyde
BAA	boron-dipyrromethene-aminoacetate
DEAB	diethylaminobenzaldehyde

RA	retinoic acid
ADH	alcohol dehydrogenase
RAR	retinoic acid receptor
EpCAM	epithelial cell adhesion molecule
FACS	fluorescent-activated cell sorting
DMEM	Dulbecco's modified Eagle's medium
FBS	fetal bovine serum
PBS	phosphate buffered saline
MEMB	mammary epithelial cell basal medium
MEGM	mammary epithelial cell growth medium
EDTA	ethylenediaminetetraacetic acid
PMSF	phenylmethanesulfonyl fluoride
SDS	sodium dodecyl sulfate
PVDF	polyvinylidene difluoride
BSA	bovine serum albumin
TBST	tris-buffered saline and tween 20
qRT-PCR	quantitative real-time polymerase chain reaction
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
RQ	relative quantification
FITC	fluorescein isothiocyanate
PE	phycoerythrin
shRNA	short-hairpin ribonucleic acid
SS	side scatter

INTRODUCTION

I. A brief overview of mammary gland development

Development of the human mammary gland is a dynamic process undergoing a series of changes including stages of growth, puberty, pregnancy, lactation and regression[1]. The mammary gland is a secretory organ that is responsible for the production of milk. It is comprised of two tissue compartments, the epithelium and the stroma[1]. The epithelium consists of mammary ducts that transport milk and alveolar cells that produce milk[1]. The stroma is a region of connective tissue that is also referred to as the mammary fat pad[1]. The stroma hosts a wide variety of cell types including adipocytes, fibroblasts, blood cells and neurons[1]. As puberty commences, estrogen and progesterone are produced in a cyclical manner causing a stimulation of ductal outgrowth and side branching[1]. During pregnancy, prolactin and placental hormones direct the proliferation and development of the alveolar compartment preparing the gland for milk secretion[2]. In pregnancy, luminal secretory cells functionally differentiate to produce milk[3]. Surrounding these secretory cells is a casing of specialized contractile myoepithelial cells that aid in milk delivery[3]. This network of ducts and alveoli is encased by the stroma, acting as a supporting structure for the epithelial components of the mammary gland[3]. When lactation ceases, the loss of suckling and the resulting loss of the stimulatory prolactin signal initiates a process called involution[4]. Involution is characterized by massive cell death of luminal cells and remodels the mammary gland to a state of simple ductal structure that resembles the gland pre-pregnancy[4]. Subsequent pregnancies will initiate a new round of alveolar proliferation, maturation and lactation[4].

The profound expansion of the mammary epithelium following successive rounds of pregnancy implicates a stem cell population with extensive regenerative capacity[5].

Stem cells are characterized by the ability to self-renew, giving rise to more stem cells, and by the ability to produce the multitude of differentiated cells that comprise the mammary epithelium[5]. Experimentally, these stem cells can be identified at the molecular level by a number of markers (e.g., cell-surface proteins)[6-7], and by gene expression profiles[8-9], as well as functional assays (e.g., enzymatic activity assays, etc.)[10], to be further elucidated below.

II. The mammary epithelium is organized into a hierarchy

The concept of a self-renewing and bipotent mammary stem cell (MaSC) was first introduced by Daniel *et al*. through pivotal transplantation experiments in mice and rats; their work revealed that the structure of the mammary gland can be recapitulated from serially transplanted random fragments of the epithelium[11]. Since then there is increasing evidence to support the presence of a differentiation hierarchy in the adult mammary gland[7,12-18]. Mammary epithelial transplant experiments have successfully demonstrated that specific cell populations are capable of regenerating a fully functional mammary gland[16-17,19]. These experiments take advantage of the fact that the epithelial ducts in a 3 week old mouse are confined to the most proximal region of the mammary fat pad; the fat pad can subsequently be cleared through a process of de-epithelialization in which the original epithelial ducts are removed[1]. Cell populations isolated based on their differing cell surface marker expression can then be transplanted into the cleared fat pad where they will be exposed to the native growth factors and hormone environment[20]. Kordon *et*

al. utilized experiments involving the transplantation of random fragments of the mammary epithelium marked with the mouse mammary tumour virus (MMTV) to highlight the regenerative capability of stem cells within the mammary gland[21]. Serial transplantations of clonally derived outgrowths were able to regenerate the functional mammary gland in its entirety[21]. It has been established that not all of the different cell types found within the mammary epithelium are capable of successfully regenerating a functional mammary gland[16-17,19]. For example, cells negative for various lineage markers (Lin^-) and positive for select integrins (ex. CD29/integrin Beta1 and various Cluster of Differentiation or CD proteins) have been shown to have stem-like properties[19]. Shackleton *et al*. demonstrated through transplantation experiments that only single cells from the $Lin^-CD29^{high}CD24^+$ population were capable of regenerating a fully functional mammary gland; cells within this population had properties of multipotency and the ability to self renew, both of which are defining characteristics of MaSCs[19]. MaSCs can divide asymmetrically[22]. Asymmetric division results in a daughter cell identical to the MaSC, functioning as a mode of self-renewal that preserves the stem cell population as well as produces another daughter cell, referred to as a progenitor cell, which can eventually become a more differentiated cell type[23]. Bipotent progenitors can give rise to the cell types that define the mature epithelium of either the luminal or myoepithelial lineage (Figure 1)[24-25]. This intermediate bipotent progenitor can differentiate towards the luminal lineage that eventually produces the ductal cells that comprise the inner lining of the ductal network and the alveolar cells that form the milk producing alveolar structures characteristic of pregnancy[24-25]. A bipotent progenitor can also differentiate towards the myoepithelial lineage[24-25]; the fully differentiated myoepithelial cells form a matrix

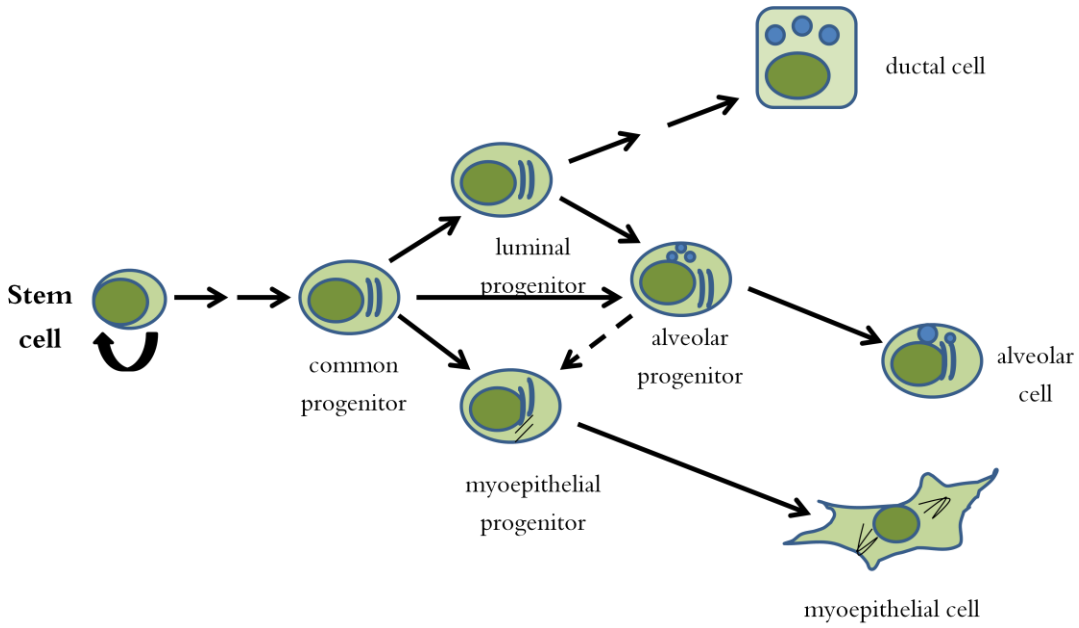

Figure 1. Schematic of differentiation hierarchy within the mammary epithelium

Mammary stem cells (MaSCs) can self-renew to produce an identical stem cell. MaSCs can also give rise to the cell types that define the mature epithelium of either the luminal or myoepithelial lineage through a common or bipotent progenitor[25]. This intermediate progenitor can differentiate towards the luminal lineage that eventually produces the ductal cells that comprise the inner lining of the ductal network and the alveolar cells that form the milk producing alveolar structures characteristic of pregnancy[1,3]. During pregnancy, the alveolar progenitor may demonstrate bipotency[25]. A common progenitor can also differentiate towards the myoepithelial lineage eventually forming fully differentiated myoepithelial cells. Myoepithelial cells form a matrix enveloping luminal secretory cells and aid in milk delivery due to their contractile nature[1,3]. Figure adapted from Visvader 2009[25].

enveloping luminal secretory cells and aid in milk delivery due to their contractile nature[3,24].

III. Stem cells have defining characteristics

Stem cells are defined by the ability to self-renew and give rise to progeny that can differentiate into the many cell types that comprise a mature gland[23]. The ability to self-renew is indicative of a stem cell's high proliferation potential and contribution to organogenesis[26]. The ability to self-renew is also critical for maintaining the mature adult gland and in some tissues contributes to repair upon insult or injury to part of the tissue[27-29]. MaSCs are critical for normal organ development, the maintenance of tissue homeostasis, and the regeneration of a functional mammary gland during successive reproductive cycles[1,5,30]. Stem cells are also defined by the ability to differentiate; this multipotent nature allows for the production of the variety of differentiated cell types that contribute to the functionality of the mature gland[5-6,30]. Stem cells have active anti-apoptotic pathways and telomerase activity that contribute to their long-lived nature[31-34]. Consequently, stem cells have more exposure to damaging agents with the risk of acquiring mutations and have developed mechanisms to increase their resistance to various damaging agents[30,32,35]. One mechanism to counteract this risk is the increased expression of membrane transporter proteins, such as P-glycoproteins or breast cancer resistance proteins[36-37]. Increased membrane transporter activity serves to protect stem cells from toxic agents by pumping potential toxins out of the cells[36-37]. It has also been suggested that membrane transporters may prevent stem cells from being subjected to differentiation cues[38]. Good *et al.* demonstrated in *Dictyostelium* that transporters function to exclude various differentiating factors, helping the stem cells remain in an

undifferentiated state[38]. In addition, stem cells are able to grow in anchorage independent conditions allowing for migration and homing to distant sites[30,39-42].

Stem cells are able to divide symmetrically and asymmetrically (Figure 2)[23]. Symmetric division involves the production of two daughter cells identical to the parent stem cell[23]. Asymmetric division occurs when the parent stem cell produces one daughter stem cell and one differentiated cell[23]. Symmetric cell division provides a mechanism for stem cells to rapidly expand in number during critical times, such as in specific developmental time periods or in response to injury[26-29]. It has been suggested that asymmetric cell division may serve as a mechanism for maintaining appropriate numbers of progeny[23]. Investigative studies into pathways regulating self-renewal decisions have revealed a role for the Notch transmembrane receptor proteins[43]. In mammals, the Notch family consists of four homologues (Notch 1 to 4)[44-47]. Notch proteins interact with both surface bound and secreted ligands (Delta, Delta-like, Jagged 1 and 2)[48], and subsequent Notch signaling is modulated by members of the fringe family[49]. Notch receptor activation involves cleavage events mediated by proteases of the ADAM (a disintegrin and metalloproteinase) family in addition to an intramembrane cleavage event mediated by presenilin[48,50]. The Notch intracellular domain then translocates to the nucleus where it can regulate gene expression of several downstream targets by interacting with a transcription factor complex comprised of C promoter binding factor (CBF), Suppressor of Hairless and Lag-1[48]. Activation of the Notch pathway regulates cell fate[51-53]. For example, over-expression of activated Notch 4 in culture serves as a block for differentiation of normal breast epithelial cells[52]. *In vivo* studies utilizing transgenic mice over-expressing activated Notch 4 in the mammary gland revealed a failure to develop

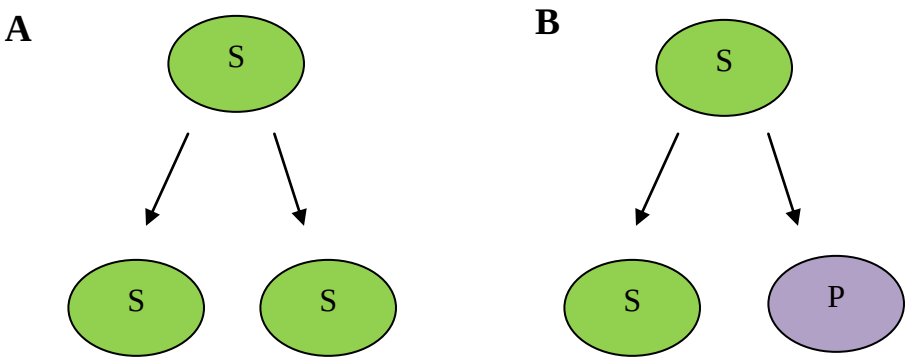

Figure 2. Modes of stem cell division

(A) Stem cells (S) can symmetrically divide producing two daughter cells identical to the parent stem cell. (**B)** Asymmetric division occurs when the parent stem cell produces one identical daughter stem cell and one differentiated progeny cell (P)[23].

normally; in addition, these mice eventually developed poorly differentiated mammary tumours[53].

IV. Cell cycle mechanisms regulating cell populations

For a cell to create a new cell containing genetically identical material it must undergo an orderly sequence of events in which it duplicates the cellular contents and subsequently divides in two; this process of sequential duplication and division events is known as the cell cycle[54]. The cell cycle is defined by distinct phases; S phase of the cell cycle is when DNA replication occurs through chromosome duplication and M phase is when mitosis and cytokinesis occur resulting in nuclear and cytoplasmic division respectively[54]. Gap phases, G_1 and G_2, provide time delays to allow for cell growth and the opportunity to monitor internal and external environmental conditions[54]. These gap phases ensure conditions are favourable before committing to DNA replication. Critical to the regulation of cell-cycle control are a type of protein kinases known as cyclin-dependent kinases (Cdks). The activities of Cdks are up-regulated and down-regulated during cell cycle progression causing orderly changes in the phosphorylation of intracellular proteins that regulate cell cycle events[54-55]. The most critical regulators of Cdk activity are proteins known as cyclins[54-55]. Cyclins have structural and functional similarities, and interact with Cdks through a conserved region of amino acids termed the cyclin box[57]. Cdks depend on cyclin binding for initiation of protein kinase activity[54-55]. In the absence of cyclin binding, a Cdk's active site is blocked by the T-loop, rendering it inactive[58]. Upon cyclin binding, the T-loop leaves the active site, resulting in the Cdk becoming partially activated[58]. Complete activation of the cyclin-Cdk complex occurs when a Cdk-activating enzyme (CAK) phosphorylates a threonine residue, causing a

conformational change[59]. The activated Cdk is then ready to phosphorylate target proteins[59]. Various cyclin-Cdk complexes form throughout the distinct phases of the cell cycle. For example, Cyclin D forms a complex with Cdk4 or Cdk6 in G_1, Cyclin E forms a complex with Cdk2 in G_1/S, Cyclin A forms a complex with Cdk2 or Cdk1 in S, and Cyclin B forms a complex with Cdk1 in M[55].

V. Mechanisms regulating Cdk activity

Additional mechanisms serve to regulate Cdk activity throughout the cell cycle. Phosphorylation of two amino acids found in the active site of the kinase, namely Thr14 and Tyr15, by the Wee1 protein kinase results in inhibition of Cdk activity[54]. Removal of this inhibitory phosphorylation state by the Cdc25 phosphatase in turn increases Cdk activity[60]. Binding of Cdk inhibitor proteins (CKIs) negatively regulates cyclin-Cdk complexes[61]. One group of CKIs is called the Cip/Kip (Cdk inhibiting protein) family and includes $p27^{Kip1}$, $p21^{Cip1}$ and $p57^{Kip2}$. Structural studies revealed that the Cip/Kip CKIs bind cyclin-Cdk complexes at the interface of the complex, obstructing the adenosine triphosphate (ATP) region of the Cdk[61]. This in turn prevents activation by obstructing proper folding of the catalytic cleft[61].

VI. MaSC quiescence

Adult stem cells are often found in a reversible state of cell cycle arrest termed quiescence[62-64]. Characterized by relative inactivity and low division rates, quiescence protects stem cells from damage to genetic material and prevents exposure to differentiation signals[62-64]. Quiescence also serves as a protective mechanism to prevent premature depletion of the stem cell population, preserving their long life span[62-64].

Quiescence is controlled at the G_1 phase of the cell cycle through the action of CKIs such as $p27^{Kip1}$, $p21^{Cip1}$ and $p57^{Kip2}$ [65-67]. When a cell receives signals to proliferate or differentiate, the actions of CKIs are inhibited, and the stem cell is free to re-enter the cell cycle[65-67]. Shackelton *et al*. demonstrated that there is a population of label-retaining cells found within populations enriched for MaSCs, suggesting a subset of quiescent cells[19]. The mammary gland niche, or microenvironment of supporting cells and extracellular elements found in the stroma, also plays a role in regulating MaSC activity[3]. It is suggested that the mammary niche provides both positive and negative signals to modulate MaSC activity[68-69].

VII. Atypical cell cycle regulators: Spy1

Xenopus Speedy was discovered through a screen for genes that displayed resistance to a *rad1* deficient strain of *Schizosaccharomyces pombe* when subjected to UV or gamma irradiation[70]. An independent group also identified a novel protein, p33-RINGO (Rapid Inducer of G_2/M progression in Oocytes) that was structurally identical to *Xenopus* Speedy[71]. p33-RINGO allowed for initiation of *Xenopus* oocyte maturation to occur and down-regulation of endogenous p33-RINGO inhibited progesterone-induced maturation[71]. *Xenopus* Speedy and the human homolog SpeedyA1 (Spy1) possess 40% homology[72]. Spy1 is encoded by the SPDYA gene on chromosome 2 in humans[73]. Spy1 is a member of the Speedy/RINGO family of proteins and the defining feature of family members is a conserved core region termed the Speedy/RINGO box that facilitates interaction with Cdks[73].

Spy1 is capable of binding and activating Cdk1 (G_2/M) and Cdk2 (G_1/S) to allow for progression through the cell cycle[70-74]. Spy1 does not display sequence homology to cyclin proteins and activates Cdks in a unique manner[74]. Unlike classical cyclins, Spy1 activates both Cdk1 and Cdk2 independent of the well defined changes in Cdk phosphorylation; Spy1 can activate Cdk1 and Cdk2 without the phosphorylation on Thr161 and Thr160 respectively[74]. In addition, Spy1-Cdk complexes are less sensitive to CKI inhibition mediated specifically through $p21^{Cip1}$ [74]. Spy1 has direct interactions with $p27^{Kip1}$ to promote its degradation; Spy1-Cdk2 complex phosphorylates $p27^{Kip1}$ at Thr187, tagging it for proteasomal degradation and allowing for cell cycle progression to occur[72,75]. Thus, Spy1 acts to enhance cell proliferation[72]. Spy1 is a nuclear protein with peak expression in the G_1/S phase of the cell cycle[75]. Therefore, Spy1 is an atypical cell cycle regulator, operating in a manner different from cyclins.

Spy1 expression is found in a multitude of human tissues, cell lines, and cancers[76-80]. Spy1 protein and RNA levels are tightly regulated during mammary gland development, showing elevated expression in the proliferating virgin gland and maintaining high levels throughout early pregnancy[78]. Spy1 levels decrease significantly in the later stages of pregnancy when terminal differentiation of the gland occurs[78]. Previous work has established a role for Spy1 in various cancers[76,79-81]. Spy1 protein levels are elevated in multiple types of glioma and are associated with increasing tumour grade[80]. Spy1 protein levels are also significantly elevated in many human breast cancers and play a role in non-hodgkin's lymphomas[81]. Recent work from the Porter laboratory has established a role for Spy1 in maintaining stemness in the brain[80]. Spy1 over-expression disrupts neuronal differentiation and promotes neurosphere clonal growth[80]. It

was also demonstrated that Spy1 plays a role in maintaining symmetric division and self-renewal of brain tumour-initiating cells (BTICs), which share many characteristics with neural stem cells[80].

VIII. The prevalence of breast cancer in Canada

In Canada, breast cancer is the most common cancer in women excluding non-melanoma skin cancers and is the second leading cause of death[82]. Estimates project that on average 24 400 Canadian women will be diagnosed with breast cancer in 2014[82]. Strikingly, this disease will claim the lives of approximately 14 Canadian women every day[82]. The effects of this disease are both devastating and widespread. Over 99% of cases affect women, suggesting a critical link between the development of the female mammary gland and the incidence of this disease[82]. Although advances in earlier detection, diagnosis and treatment have given hope to those diagnosed with this disease, much work remains to be done in the fight against breast cancer. Breast cancer is an extremely heterogeneous disease, with stark differences at both the histological and molecular levels. Gene expression profiling has identified at least six different subtypes of breast cancer[8-9]. The subtypes include luminal A or B, basal-like, claudin-low, human epidermal growth factor receptor 2 over-expressing (HER2/ERBB2), and normal-breast-like (Table 1)[8-9]. It is hypothesized that the different subtypes may be reflective of different cells of origin responsible for initiating tumour formation[83-84]. The different subtypes may also reflect differences in mutational profiles[8]. There is controversy over whether normal-breast-like is a distinct molecular subtype; this subtype accounts for less than 10% of all breast cancers, typically is characterized by small tumours and has a favourable prognosis[85-86]. Luminal cell differentiation is associated with luminal A and B

Table 1: Breast cancer subtypes

Classification	Receptor status	Ki67 status	Response to therapy	Potential cell of origin	Representative cell line
Luminal A	ER^{+} $PR^{+/-}$ $HER2^{-}$	low	often chemotherapy responsive	differentiated luminal cells	MCF-7
Luminal B	ER^{+} $PR^{+/-}$ $HER2^{+}$	high	variable chemotherapy response, endocrine responsive	differentiated luminal cells	BT474
HER2	ER^{-} PR^{-} $HER2^{+}$	high	chemotherapy responsive, trastusumab responsive	late luminal progenitor	SK-BR-3
Basal	ER^{-} PR^{-} $HER2^{-}$	high	endocrine nonresponsive, variable chemotherapy response	bipotent progenitor/luminal progenitor	MDA-MB-468
Claudin-low	ER^{-} PR^{-} $HER2^{-}$	low	low chemotherapy response	mammary stem cell	MDA-MB-231

subtypes; these subtypes are usually responsive to therapies and thus associated with favourable patient outcome[25,84,87-88]. Breast cancers over-expressing HER2 also exemplify luminal characteristics, although this subtype has poor patient survival rates[84,87-89]. Basal breast cancers encompass 15-20% of all breast cancers, are heterogeneous in nature, and are poorly differentiated[90]. Claudin-low breast cancers characteristically have decreased expression of claudins, proteins involved in tight-junctions and cell-to-cell adhesion[91]. Another approach to stratifying breast cancers is based on receptor status; breast cancers can be classified based on the presence or absence of the estrogen receptor (ER), progesterone receptor (PR) and amplification of the HER2/ERBB2 locus[92-94]. Stratification of breast cancers based on receptor status allows prediction of a probable response to specific therapies and has improved predictions of overall patient outcome[89,92,94]. The most aggressive tumours are classified as triple negative, referring to the lack of expression of ER, PR, and HER2 and typically respond poorly to treatment[94]. However, despite increases in predictability based on receptor status of tumours, patient response to chemotherapy still varies substantially[25]. Improving detection and treatment options for breast cancer patients ultimately requires a complete understanding of the specific populations of cancer cells that actively drive breast tumourgenic growth.

IX. The cancer stem cell model

The cancer stem cell (CSC) model is based on a hierarchical model of tumour development[32]. It suggests that only a small population of cells is capable of initiating tumours and the vast majority of cells within a tumour are differentiated with limited replicative potential[32,95]. The CSC model hypothesizes that deregulation of processes

governing normal adult stem or progenitor cells results in malignant transformation of this population of cells, allowing for them to drive tumour growth and progression[32,95]. Clonal expansion of the stem and progenitor populations allows for the possibility of accumulating additional genetic or epigenetic changes, resulting in complete malignant transformation of these cells[32,95]. It is this dangerous population of CSCs that initiate and drive tumour progression[32,95]. This is in contrast to the stochastic model of tumour development, which argues all cells within a heterogeneous population have the capacity to initiate a tumour[32]. Evidence for the existence of a CSC was solidified in 1994 by Dr. John Dick in a leukemia model system[96]. This pioneering study revealed that cells expressing a $CD34^{+}CD38^{-}$ cell surface phenotype were leukemia-initiating cells; when injected into severe combined immunodeficient (SCID) mice these cells were able to form tumours that resembled the heterogeneous tumours found in acute myeloid leukemia patients[96]. Evidence for the existence of CSCs in solid tumours was demonstrated by the observation that not all cell types within breast tumours were capable of initiating tumour growth when transplanted into immunodeficient mice[97]. Breast cancer cells marked with the cell surface marker phenotype $CD44^{+/high}/CD24^{-/low}$ have stem-like properties and enhanced tumourigenic capacity[97]. CD24, also known as heat specific antigen, is a glycosylphosphatidylinositol-anchored glycoprotein involved in cell adhesion[98]. CD44 is a transmembrane glycoprotein involved in numerous cellular processes such as cell migration, homing and adhesion[99]. Al-Hajj *et al*. isolated breast cancer cells based on a $CD44^{+}CD24^{-}$ phenotype from primary tumours and pleural effusions of breast cancer patients and injected them into cleared fat pads of immunocompromised mice[97]. As few as 100 $CD44^{+}CD24^{-}$ cells formed tumours, whereas

injection with over 10 000 $CD44^{-}CD24^{+}$ cells did not[97]. Numerous studies have provided support for the concept that not all cells within a tumour are created equal in terms of their tumour forming ability and capacity to recapitulate a heterogeneous tumour[97,100-102].

Analysis of both normal stem cells and CSCs reveals many similarities in important phenotypic characteristics[30]. Normal adult stem cells are slow-dividing and long-lived, the latter an attribute which increases the risk of accumulating mutations to serve as a target for transformation[30]. A normal adult stem cell is in part defined by its ability to self-renew, a property CSCs may use to achieve uncontrolled proliferation and tumourgenicity[30]. A CSC is able to differentiate into the multitude of cell types that comprise a tumour, contributing to tumour heterogeneity[30]. Zucchi *et al*. showed that a single LA7 cell derived from rat mammary adenocarcinoma was able to differentiate into all the cell lineages found within the mammary gland[102]. Normal adult stem cells have enhanced protective mechanisms against toxic insults; similarly, CSCs may be resistant to damaging agents and may serve as one explanation for chemoresistance in clinical settings[30]. Normal adult stem cells are typically anchorage-independent, with the ability to survive and migrate to distant sites[30]. This feature may be exploited by CSCs to achieve metastasis, or the development of a malignant growth at sites distant from the primary tumour[30]. The CSC model for carcinogenesis carries significant clinical implications, as this aggressive population of cells may be protected against the action of conventional therapies and serve as a mechanism for relapse[30,32,95].

X. Mammosphere culture as a tool to enrich for mammary stem, progenitor, and breast CSCs

Epithelial cells depend on interaction or attachment to a substratum when cultured to survive and proliferate; that is, normal epithelial cells are anchorage-dependent and undergo apoptosis when unable to attach to a substratum[39-42,103]. The mammosphere assay takes advantage of the observation that stem cells are able to grow in serum-free suspension, which *in vivo* allows for migration and homing to distant sites[30,104-105]. Based on the model of neurospheres (free-floating spherical structures enriched for neural stem and progenitor cells)[40], a culture system was developed that involved seeding human mammary epithelial cells onto ultra-low attachment plates in order to enrich for cells able to grow in anchorage-independent conditions[106]. Early mammosphere experiments revealed that a small subset of cells are able to survive and proliferate in such conditions, forming multicellular spheroids termed 'mammospheres'[106-107]. Dontu *et al*. demonstrated that mammospheres are enriched for bipotent progenitors eightfold over mammary cells grown in anchorage-dependent conditions[106]. They further demonstrated that these progenitors could differentiate into myoepithelial, ductal or alveolar cells[106]. When subjected to 3 dimensional culture systems, progenitors were able to form complex functional structures[106]. Self-renewal properties of the different cell types forming mammospheres were also assessed through clonal assays in which mammospheres were dissociated into single cell suspensions, re-plated, and tested for the ability to form second generation spheres[106]. The results support the model of a MaSC undergoing limited self-renewal divisions and giving rise to more differentiated progenitors[106]. Microarray analysis revealed differences in the gene expression profiles of multipotent

cells in secondary mammospheres compared to cells grown in conditions favouring differentiation[106]. Genes expressed in mammospheres highly overlapped with genes expressed in haematopoietic, neuronal and embryonic stem cells[106]. For example, increased active TGF-beta signalling and increased expression of membrane transporter proteins were found in mammospheres[106]. These characteristics are consistent with previously established stemness attributes[106,108]. Thus, the mammosphere assay is a reliable *in vitro* suspension culture system that allows for the study and enrichment of mammary stem and progenitor cells.

XI. Aldehyde dehydrogenase as a marker for normal and CSCs

The human aldehyde dehydrogenase (ALDH) superfamily encompasses 19 known putatively functional genes[109-110]. ALDH enzymes show multiple areas of localization including in the cytosol, nucleus and mitochondria and vary widely in their tissue and organ distribution[111-113]. The ALDH superfamily is a group of enzymes that catalyze the oxidation of aldehydes to their corresponding carboxylic acids[109-110,112]. Aldehydes are long-lived, highly reactive compounds with critical roles in normal physiological responses, and with mutagenic and cytotoxic potential[109,112]. Aldehydes can come from both endogenous and exogenous sources. Endogenous aldehydes are generated through metabolic amino acid catabolism[112], metabolism of vitamins and steroids[109-110], in addition to several other metabolic processes. Exogenous aldehydes can be generated through biotransformation of xenobiotics and drugs, and are present in smog, cigarette smoke and motor vehicle exhaust[112]. Therefore, ALDH enzymes play a critical role in protecting cells from the possible detrimental effects of endogenous and exogenous aldehydes[112]. It has been shown that the ALDH1 family (ALDH1A1, 1A2,

1A3, 1L1, 1L2) are highly expressed in adult stem cells and CSCs and thus are used as markers to characterize this distinct population[10, 112]. To avoid pitfalls with enzyme kinetics and immunoblotting methods which require lysis and endogenous release of ALDH enzymes from cells[114-115], the use of flow cytometry and fluorescent substrates for ALDH1 allows for the study of ALDH1 activity in viable cells[116-117]. Storms *et al.* developed an assay (Aldefluor® Assay) in which a fluorescent ALDH1 substrate, BODIPY aminoacetaldehyde (BAAA) passively diffuses into intact, viable cells (Figure 3)[117]. ALDH1 will subsequently convert BAAA into the negatively charged product BODIPY-aminoacetate (BAA^-)[117]. BAA^- is trapped inside the cell and consequently, cells with high ALDH1 activity become highly fluorescent[117]. Use of cold assay buffer prevents the ATP-binding cassette transporters from excluding the BAA^- substrate out of the cells[10]. To distinguish cells with high ALDH1 activity, populations in the top 10-20%, populations are compared to a negative control utilizing diethylaminobenzaldehyde (DEAB), a specific inhibitor of ALDH1[117-119]. The Aldefluor® Assay serves as a reliable tool to identify cells with high ALDH1 activity in various human models[118-120].

Cancer cells expressing high levels of ALDH activity have increased tumourigenic capacity and demonstrate more stem-like characteristics compared to low ALDH expressing cells[118-119]. Ginestier *et al.* used transplantation experiments to demonstrate the highly tumourigenic nature of $ALDH^+$ cells[119]. When 50 000 $ALDH^-$ cells were transplanted into cleared fat pads of immunocompromised mice no tumours developed; when 500 $ALDH^+$ cells were transplanted tumours formed within a 40 day time period[119]. Recent evidence suggests high activity of ALDH is associated with poor prognosis in breast, bladder and prostate cancer patients[119,121-122]. Specific to breast

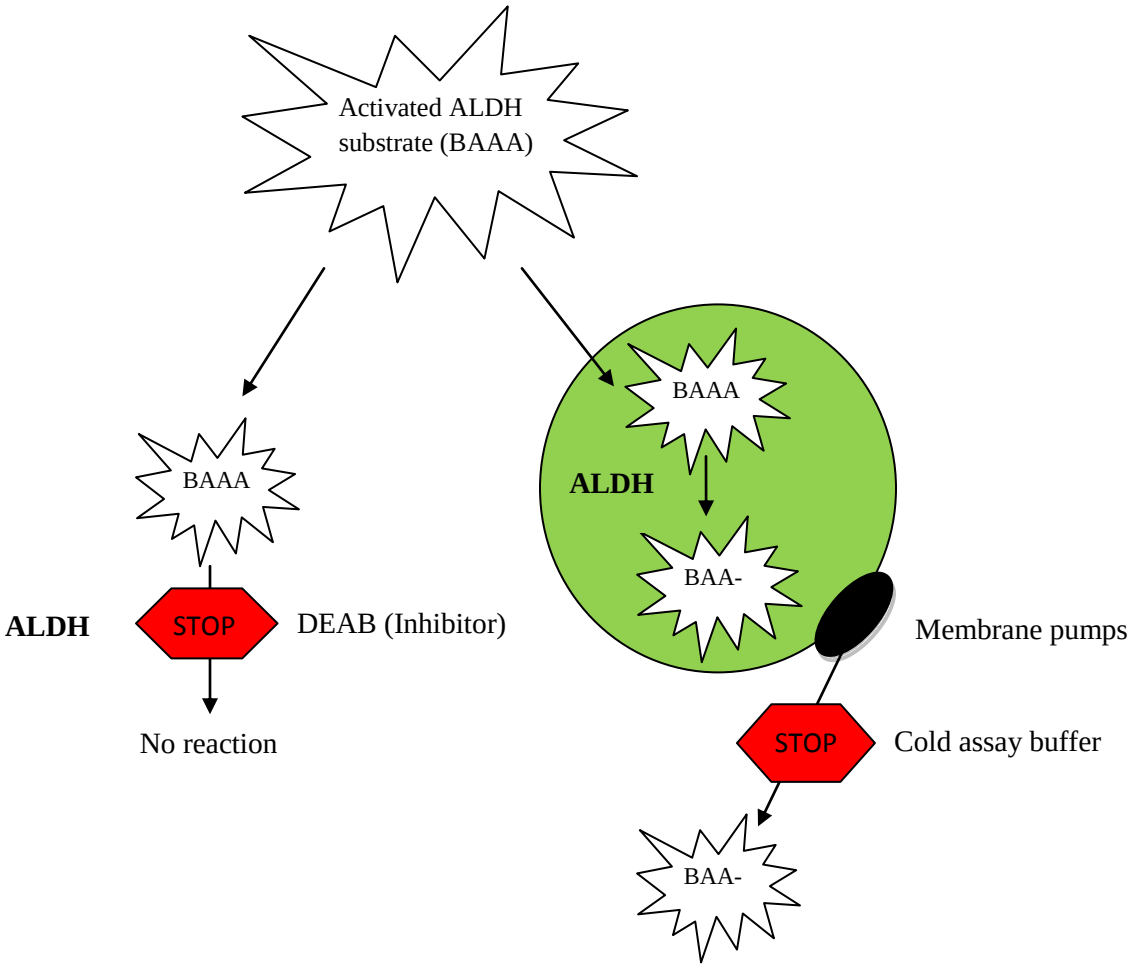

Figure 3. Schematic of Aldefluor® Assay

The Aldefluor® Assay serves as a reliable tool to indentify cells with high ALDH activity in various human models[118-120]. A fluorescent substrate for ALDH1, BODIPY aminoacetaldehyde (BAAA), passively diffuses into intact, viable cells. ALDH1 will subsequently convert BAAA into the negatively charged product BODIPY-aminoacetate (BAA^-). BAA^- is trapped inside the cell and consequently, cells with high ALDH activity become highly fluorescent. Use of cold assay buffer prevents the ATP-binding cassette transporters from excluding the BAA^- substrate out of the cells. To distinguish cells with high ALDH activity, populations are compared to a negative control utilizing diethylaminobenzaldehyde (DEAB), a specific inhibitor of ALDH1. Figure adapted from Aldefluor® Assay Information Sheet (www.stemcell.com)

cancer, a study analyzing the tumours of 577 patients revealed tumours positive for ALDH have a significantly lower survival overall compared to patients with tumours negative for ALDH[119]. It is important to note that ALDH can serve as a valid CSC marker in tissue types that normally do not express high levels of ALDH[123]. Some of these tissues include the breast, lung and colon[123]. However, tissues with normally high levels of ALDH, such as liver and pancreas, are not suitable for this type of analysis[123].

Perhaps the most established functional role of ALDH in cell populations is in the retinoid signalling pathway. Retinoic acid (RA) has established roles in regulation of gene expression, morphogenesis and development[124-126]. Retinol is oxidized by alcohol dehydrogenase (ADH) into retinaldehyde; this is a reversible reaction[112,124]. Retinaldehyde is then irreversibly oxidized into RA by ALDH1. RA is then free to bind the retinoic acid receptor (RAR) mediating changes in gene expression and cell differentiation[124,127]. The regulation of ALDH1 is controlled by a negative feedback mechanism[127]. Another functional role for the ALDH superfamily is that of detoxification and cellular protection[109-112] and mutations and overall deficiencies in specific ALDH enzymes are associated with disease states[128-129]. For example, mutations in ALDH1A2 are associated with spina bifida and ALDH2 with hypertension[128-129]. Using the hematopoietic model, it was elegantly demonstrated that cells with high ALDH activity were resistant to cyclophosphamide, a potent alkylating agent[130]. Using mouse models, it was found that inhibiting the activity of ALDH1 caused a delay in the G_o/G_1 transition, causing more hematopoietic stem cells to accumulate in G_o compared to G_2/S/M phases[131]. This has powerful clinical implications. Targeting cells expressing high levels of ALDH towards a more differentiated state may make them more sensitive to

conventional therapies[132]. Similarly, targeting ALDH with DEAB can result in stem cell expansion and can be used in applications like bone marrow transplants to improve engraftment and patient survival[131]. More research is needed to investigate these potential clinical avenues.

XII. Cell surface marker expression can be used to isolate stem, progenitor and breast CSCs

Another method researchers use to isolate MaSCs is separating sub-populations based on the cell surface marker expression of the different cell types found within the mammary epithelium[17,19]. Primary cell surface marker phenotype differs in the isolation of mouse and human MaSC, although there are some instances of overlap[25]. For example, MaSCs can express high levels of alpha 6 (CD49f) and/or beta 1 (CD29) integrins; mouse MaSCs are enriched in the $CD49f^{high}CD29^{high}$ population whereas human MaSC are enriched in the $CD49f^{high}CD24^{-}EpCAM^{-/low}$ subset[24-25]. Beta 1 integrin is an important extracellular matrix receptor that acts as a heterodimer with alpha and beta subunits[133]. A role for beta 1 integrin has been established in the mammary gland, as it helps in maintaining the stem cell pool and regulates the balance between basal and luminal lineages through interactions with the stem cell environment[133]. Mammary tumours often display decreased expression of both alpha 6 and beta 1 integrin[134-135]. It is suggested that this down-regulation may allow for stem cells to detach from their native microenvironment and migrate to other areas[134-135]. Researchers can use cell surface marker expression in a combinatorial manner to isolate specific populations, sort these populations based on fluorescence-activated cell sorting (FACS), and complete further analysis for stemness properties both *in vitro* and *in vivo*[97,137]. As previously mentioned,

human breast cancer cells marked with the cell surface marker phenotype $CD44^{+/high}/CD24^{-/low}$ have stem-like properties and enhanced tumourigenic capacity[97,137]. Clarke *et al*. isolated cells based on a $CD44^{+}CD24^{-/low}Lin^{-}$ phenotype; injecting 200 of these cells into cleared mammary fat pads of immunocompromised mice resulted in a heterogeneous tumour whereas 20 000 cells negative for this phenotype did not[137]. $CD44^{+}CD24^{-/low}Lin^{-}$ cells retained tumourigenic ability after serial passaging, highlighting their ability to self-renew[137]. Al-Hajj *et al*. isolated breast cancer cells based on a $CD44^{+}CD24^{-}$ phenotype from primary tumours and pleural effusions of breast cancer patients and injected them into cleared fat pads of immunocompromised mice[97]. As few as 100 $CD44^{+}CD24^{-}$ cells formed tumours[97]. Expression profiling of claudin-low tumours reveals significant overlap with the $CD44^{+}CD24^{-/low}$ breast cancer stem cell population[137]. It has also been shown that cells with a $CD44^{+}CD24^{-}$ phenotype exhibit enhanced invasive properties that may contribute toward metastatic success and express higher levels of anti-apoptotic proteins[138]. *In vitro* experiments revealed only the $CD44^{high}CD24^{low}$ fraction of the population are capable of forming mammospheres[106]. Although it is well established in the literature that this cell surface phenotype enriches for stem and progenitor cells, it likely does not solely contain only CSCs. Using this phenotype in combination with other markers, such as $ALDH^{+}$, may represent the most aggressive CSC population[119,132].

XIII. Using breast cancer cell lines as an *in vitro* model to study breast cancer

Breast cancer cell lines are a valuable *in vitro* tool for researchers to dissect molecular mechanisms regulating the growth of breast cancer[139]. Neve *et al*. assessed the molecular and biological similarities and differences between 51 breast cancer cell lines

and primary human breast tumours[140]. Comparing genomic features, cell lines display the same heterogeneity in copy number and expression aberrations as do primary tumours[140]. In addition, cell line karyotypes remain relatively stable during extended culture exposure[140]. Comparison between transcription profiles revealed that breast cancer cell lines cluster into basal-like and luminal expression subtypes similar to primary tumours[140]. However, tumours clearly resolve into two luminal subsets, which are less apparent in cell lines[140]. Similarly, cell lines distinctly resolve into Basal A and Basal B clusters, which are less apparent in primary tumours[140]. This may be due to the absence of stromal interactions and/or the lack of native physiological interactions that exist in the primary tumour microenvironment[141]. It has also been demonstrated that cell lines contain functional CSCs[142]. Within 23 different breast cancer cell lines, the ALDH positive population was sorted and subjected to analysis for stemness properties *in vitro* and *in vivo*[142]. It was demonstrated that ALDH positive cells isolated from cell lines were able to form mammospheres in culture, as well as form tumours when injected into immunodeficient mice[142]. Overall the vast majority of breast cancer cell lines accurately reflect the genomic and transcriptional characteristics of primary breast tumours and provide a convenient tool for researchers to dissect mechanisms regulating breast cancer initiation and progression[140,142].

XIV. The role of Spy1 in breast cancer

A potential role for Spy1 in breast cancer first emerged when Zucchi *et al*. found Spy1 as one of the 50 genes over-expressed in breast ductal carcinoma[76]. *In vivo* transplantation experiments revealed that Spy1 over-expressing HC11 cells can accelerate tumour formation in the mammary gland[78]. High Spy1 levels are found in

aggressive breast cancers and down-regulation of Spy1 significantly inhibits breast cancer cell growth[79]. It has also been shown that Spy1 protein levels are elevated in human breast cancer cell lines[79]. Taking into account the established role of Spy1 in breast cancer and maintaining stemness characteristics in other systems[80], I sought to investigate the potential role of Spy1 in the CSC and progenitor populations in breast cancer through a variety of reliable *in vitro* techniques. I hypothesize that **Spy1 plays an important role in the cell cycle regulation of breast cancer stem and/or progenitor cells.**

Objective 1: Determine a role for Spy1 in driving breast cancer stem and/or progenitor cell growth. Various breast cancer cell lines were utilized as a model system of breast cancer, reflective of some of the different subtypes of breast cancer. The essentiality of the Spy1 protein was tested by manipulating levels (over-expression and knock-down) of Spy1 by lentiviral infection. The effect on the relative stem cell population was assessed through mammosphere assays and cell surface marker analysis via flow cytometry.

Objective 2: Study the functional effect of Spy1 manipulation on the breast cancer stem and/or progenitor cell populations. Using breast cancer cell lines as a model system, Spy1 protein levels were manipulated by lentiviral infection and the relative effect on the ALDH positive population was tested via flow cytometry analysis.

The CSC model for carcinogenesis carries significant clinical implications, as cancer stem cells have enhanced protective mechanisms that make them resistant to conventional therapies[30,32,35]. Designing treatment options to target this aggressive

population requires an understanding of the mechanisms regulating their cell growth and fate decisions. The cell cycle lies at the heart of these decisions, however there are large gaps in knowledge regarding how this occurs. This research aims to resolve the key cell cycle mediators, namely Spy1, in regulating specific breast cancer stem and/or progenitor cell decisions, work that may be essential for advancing potential treatment options and preventing patient relapse.

MATERIALS AND METHODS

I. Cell lines utilized

The human breast cancer cell lines used are listed in Table 2.

II. Cell culture

MDA-MB-231 were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco 12483) and 1% penicillin and streptomycin (Gibco 1540). Once cells reached confluency, plates were washed with sterile 1XPBS and 1mL of 0.05% trypsin (HyClone SH3023601) was added to the plate for 3-4 minutes. Cells were then collected by centrifugation for 5 minutes at 1000rpm. Cells were cultured in a 5% CO_2 environment.

MCF7s were maintained in DMEM supplemented with 10% FBS and 1% penicillin and streptomycin. Once cells reached confluency, plates were washed with sterile 1XPBS and 1mL of 0.05% trypsin was added to the plate for 3-4 minutes. Cells were then collected by centrifugation for 5 minutes at 1000rpm. Cells were cultured in a 5% CO_2 environment.

SK-BR-3 cells were maintained in McCoy’s 5A media (ATCC 30-2007) supplemented with 10% FBS and 1% penicillin and streptomycin. Once cells reached confluency, plates were washed with sterile 1XPBS and 1mL of 0.25% trypsin was added to the plate for 3 minutes. Cells were then collected by centrifugation for 5 minutes at 1000rpm. Cells were cultured in a 5% CO_2 environment.

III. Establishment of stable cell lines

10 000 cells per well were seeded in a 96 well plate containing 500µl of DMEM media supplemented with 10% FBS for MCF7 and MDA-MB-231 cells and 500µl McCoy's 5A media with 10% FBS for SK-BR-3 cells, in the absence of penicillin and streptomycin. Cells were grown overnight in a 5% CO_2 environment. The following day the growth media was changed to 500µl DMEM or McCoy's 5A containing no serum or antibiotics with 10µg/mL polybrene (Santa Cruz sc-134220). Cells were incubated for 20 minutes before virus was added to each well. The plate was gently rocked back and forth and was returned to the incubator for approximately 24 hours. Multiplicity of infection (MOI) was 10 and the virus titer for both control and shSpy1 was 10^7 titer units (TU). After 24 hours, virus was removed by aspirating the media and replaced with fresh growth media. The empty vector control (pLKO) and Spy1 knock-down (shSpy1) contained puromycin selection and thus fresh media containing 10µg/mL puromycin (Sigma-Aldrich P8833) was used to select for successfully infected cells and changed every 2 days.

Over-expression of Spy1 (pEIZ-Spy1) or control (pEIZ) in MDA-MB-231 and MCF7s were established by lentiviral infection using the same protocol as previous with the exception of puromycin selection. Successful infected cells over-expressing Spy1 fluoresced green and this was monitored through fluorescence microscopy beginning 1 week after infection.

IV. Mammosphere assay

Cells were seeded into 6-well ultra low attachment plates at 50 000 cells/well (Corning 07-200-601). Each well contained 2mL of mammary epithelial basal medium (MEBM, Clonetics CC-3152) supplemented with mammary epithelial cell growth medium (MEGM) Single Quots (Clonetics CC-4136), 20ng/mL human basic fibroblast growth factor (Sigma-Aldrich F0291), and 4µg/mL heparin (Sigma-Aldrich H0777). Mammospheres were grown for 7 days in a 5% CO_2 environment. Cells were imaged using the Leica CTR6500 microscope using AF software after 7 days. The field of view calculation was determined as follows: each well was divided into 4 quadrants and 3 random images were taken per quadrant for a total of 36 images for each condition to generate the average number of mammospheres formed for each condition. Experiments were repeated in triplicate. The average mammosphere diameter (µm) was calculated by taking the mean of all mammospheres imaged for each condition using ImageJ software.

V. Western blot analysis

For protein extraction, cell pellets were collected and lysed using lysis buffer (1M Tris-HCL pH 8.0, 2.5M NaCl, 0.5M EDTA pH 8.0, 2.5mL Triton X-100) supplemented with protease inhibitors Aprotinin (0.5µL/mL), Leupeptin (1µL/mL), and PMSF (10µL/mL). Protein lysates were stored at -20°C. A Bradford Assay was performed to determine protein concentrations. Briefly, a standard curve was generated and subsequently protein concentrations of samples (5µL sample to 995µL Bradford reagent) were determined using absorbance readings at 595nm on a spectrophotometer (Biomate 5 Thermo Electron Corporation BIO145108). Protein concentrations were corrected to the

lysis buffer reading. Samples were prepared using a total of 100 to 150µg of lysate combined with 4X sample buffer (10% glycerol, 62.5mM Tris-HCL pH 6.8, 2% sodium dodecyl sulfate (SDS), 0.01mg/mL bromophenol blue, and 2% beta-mercaptoethanol). Samples were run on a 10% SDS-PAGE page for 3 hours and 30 minutes at 120V (Fisher Scientfic FB200). Gels were subsequently transferred to a PVDF membrane (Millipore IPVH00010) for 2 hours 30 minutes at 30V. The membrane required methanol activation for 1 minute prior to the transfer. Membranes were blocked using 1% BSA (1g of Albumin Bovine BioBasic Canada Inc. AD0023 in 100mL TBST) for 1 hour on a shaker. Membranes were incubated with primary antibodies overnight at 4°C rotating constantly. The following primary antibodies were used: SPDYA (Abcam ab153965), cyclin E (Abcam ab33911), Numb (Cell Signalling 2756) and Actin Clone C4 (Merck Millipore mAB1501R). The next day, membranes were washed in 10 minute intervals in TBST for a total of 3 times. Membranes were submerged in secondary antibodies (anti-mouse IgG-Peroxidase and anti-rabbit IgG-Peroxidase Sigma-Aldrich A9917 and A0545 respectively) diluted in 1% BSA for 1 hour at room temperature while continuously shaking. Subsequently, membranes were washed again in 10 minute intervals in TBST 3 times. Membranes were imaged under chemiluminescence and densitometry analysis was performed using FluorChem HD2 imaging software (Alpha Innotech).

VI. Quantitative real-time polymerase chain reaction (qRT-PCR)

RNA was extracted from samples using a RNeasy Extraction Kit (Qiagen 74134). Briefly, cells were collected as pellets, lysed and vortexed followed by removal of genomic DNA. Ethanol was subsequently added to the samples to facilitate RNA binding

to a RNeasy spin column. Several wash steps were performed and RNA was eluted using RNase-free water. Concentration and purity were monitored using a NanoDrop Spectrophotometer (ND-1000 software version 3.3.0 Thermo Scientific). Reverse transcription of RNA utilized Superscript II reverse transcriptase (Invitrogen 100004925), 0.5µg Oligo dT's (Eurofin) and 0.5µg random nanomers (Thermo Scientific S0142). qRT-PCR was run on an ABI Viia7 thermocycler (Applied Biosystems 278880504) using Fast SYBR green detection (Applied Biosystems 4385616). Reactions were run over the course of 55 cycles including steps for cDNA denaturation, primer annealing to single stranded DNA, and elongation. Primers were used at a concentration of 5µM. GAPDH was used as an internal control. Primers used are listed in Table 3. RNA samples were stored at -80°C.

VII. qRT-PCR calculations

Analysis of qRT-PCR reactions was completed using Viia7 software version 1.1.5. Ct values were generated. The Ct value of the gene of interest is normalized to GAPDH which served as the internal control. This resulted in a ΔCt value (ΔCt= Ct gene of interest-Ct GAPDH). The control/calibrator, for example pLKO, is then set to 0 and all remaining samples are compared to this to generate ΔΔCt values ($\Delta\Delta Ct_{shSpy1} = \Delta Ct_{shSpy1} - \Delta Ct_{pLKO}$). The relative quantification (RQ) value is then calculated ($RQ_{shSpy1} = 2^{-\Delta\Delta CshSpy1}$). Data is displayed as $\log_{10}$ RQ, representing the fold change between the sample and the calibrator. Error bars represent the standard error of the average ΔCt value.

VIII. Cell surface marker analysis

Detection of fluorescent signals were detected using flow cytometry using the FL1 (525 BP filter detecting FITC/green) or FL2 (575 BP filter detecting PE/red) channels. PEIZ over-expression plasmids contain a zsGreen cassette and are detected on the FL1 channel. For each sample 500 000 cells were collected and stained with antibodies against CD24-PE (Abcam Inc. ab77219) or CD44-PE (STEMCELL Technologies Clone IM7 60068PE) for 45 minutes covered on ice. Controls were used to set up gates prior to running samples (positive control >90% fluorescent in FL2, 0% fluorescent in FL1). Cells without antibody treatment were used to verify the absence of non-specific signals. Approximately 200 000 cells were run per reaction. For cells with Spy1 knock-down, each sample was double-labelled with CD24-FITC (STEMCELL Technologies Clone 32D12 10424) and CD44-PE covered on ice for 45 minutes. Following the incubation period, cells were collected by centrifugation at 250 x g for 5 minutes. Cell pellets were then resuspended in 500µl cold 1XPBS and samples were immediately run on the Beckman Coulter Cytomics FC500 (SYS. ID 469005). Analysis was completed on CXP Software (Beckman Coulter).

IX. ALDEFLUOR® Assay

Aldehyde dehydrogenase detection was conducted using the ALDEFLUOR® Assay (STEMCELL Technologies 01700). As per the manufacturer's guidelines 200 000 cells were collected and resuspended in 1mL of ALDEFLUOR® Assay Buffer. For the negative control, 5µl ALDEFLUOR® DEAB Reagent was added to a 50mL conical tube and set aside. 5µl of the activated ALDEFLUOR® Reagent was added to the cell

suspension, mixed thoroughly by pipetting and subsequently 500µl was immediately transferred to the DEAB-containing control tube. Both the test and the control samples were incubated at 37°C for 45 minutes. Following the incubation period, cells were collected by centrifugation for 5 minutes at 250 x g. Cells were resuspended in 500µl ALDEFLUOR® Assay Buffer and samples were stored on ice until run on the Beckman Coulter Cytomics FC500. For data acquisition a Side Scatter versus FL1 dot plot was generated and 100 000 events were collected for each control and test sample using the same instrument settings.

X. Statistical analysis

Statistics were performed using a Student's paired *t*-test. Data was considered significant if the p-value was less than 0.05.

Table 2: Human breast cancer cell lines

Cell line	Source	Receptor Status	Classification
MDA-MB-231	ATCC	ER^-, PR^-, $HER2^-$	Claudin-low
SK-BR-3	ATCC	ER^-, PR^-, $HER2^+$	HER2
MCF7	ATCC	ER^+, $PR^{+/-}$, $HER2^-$	Luminal A

Table 3: Human qRT-PCR primer pairs

Human Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
GAPDH	GCACCGTCAAGGCTGAGAAC	GGATCTCGCTCCTGGAAGATG
Spy1	TTGTGAGGAGGTTATGGCCATT	GCAGCTGAACTTCATCTCTGTTGTAG

RESULTS

I. Manipulation of Spy1 levels affects mammosphere forming ability

To analyse the effect of elevated Spy1 levels on the relative stem and/or progenitor population in triple negative breast cancers, Spy1 protein was over-expressed in MDA-MB-231 and seeded into mammosphere culture (Figure 4). Conditions that favour the growth of stem-like cells with the ability to self-renew and differentiate were used[106-107]. To verify Spy1 levels in the heterogeneous population, western blot analysis was performed (Figure 4A and 5A). Mammospheres were imaged using bright-field and fluorescence microscopy to indicate cells were successfully expressing pEIZ and pEIZ-Spy1 throughout the duration of mammosphere culture (Figure 4B). Results indicate that Spy1 over-expression significantly increases the number of mammospheres formed by approximately 18% compared to control conditions (Figure 4C).

Since MDA-MB-231 cells express relatively high levels of Spy1, Spy1 knock-down was performed to test the endogenous significance on mammosphere formation (Figure 5A and 5B). Infected cells expressing pLKO or pLKO-shSpy1 were grown in puromycin-containing media for selection. Mammospheres were monitored using bright-field microscopy (Figure 5C) and results reveal that knock-down of Spy1 significantly decreases the number of mammospheres formed by approximately 26% compared to control conditions (Figure 5D). Spy1 knock-down did not statistically alter mammosphere diameter compared to pLKO conditions (Figure 5E). These results indicate that Spy1 manipulation in some triple negative breast cancer cells affects the number of mammospheres formed.

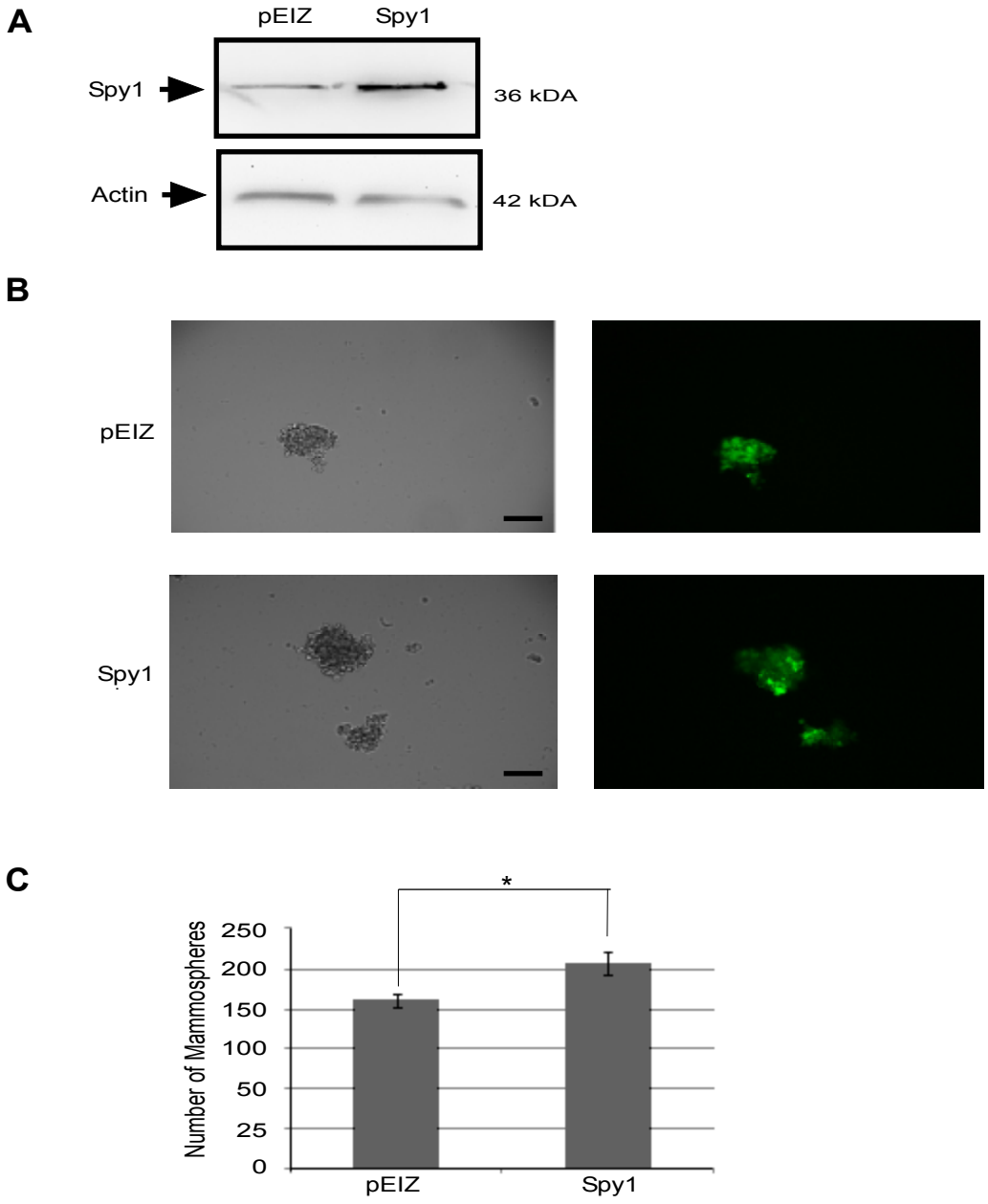

Figure 4. Over-expression of Spy1 increases the number of mammospheres formed. MDA-MB-231 cells were infected with lentiviral vectors containing pEIZ as a control and pEIZ-Spy1 to over-express Spy1. Successfully infected cells fluoresce green. **(A)** Spy1 protein levels were measured using western blot analysis. Actin served as a loading control. **(B)** Representative microscopy images showing bright-field (left panel) and fluorescent (right panel) images of mammospheres from pEIZ control and Spy1 over-expressing conditions. Total magnification of 100x. **(C)** Average number of mammospheres for pEIZ control and Spy1 conditions was determined using field of view calculation. Error bars represent standard error of the mean of three independent experiments each counted in triplicate. *$p<0.05$. Statistical significance was assessed using a student's paired *t*-test.

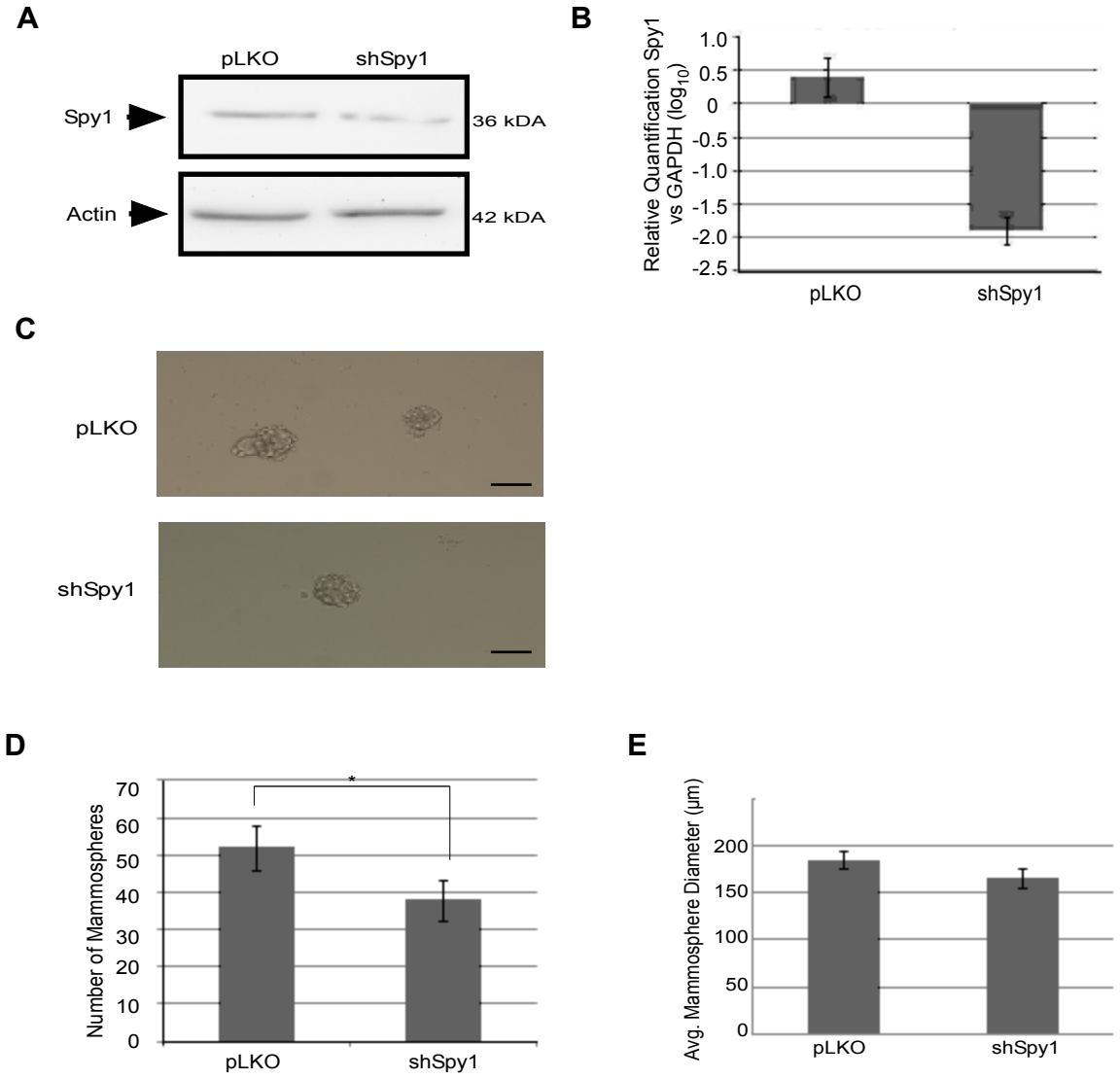

Figure 5. Spy1 knock-down decreases the number of mammospheres formed. MDA-MB-231 cells were infected with lentivirus carrying shRNA against Spy1 (shSpy1) or a scrambled control (pLKO). **(A)** Spy1 protein levels were measured using western blot analysis. Actin served as a loading control. **(B)** Efficiency of Spy1 knock-down was assessed using qRT-PCR. Data is normalized to GAPDH and presented as relative quantification (RQ) on a logarithmic scale (log10). Error bars represent standard error of the mean of two independent experiments run in triplicate qRT-PCR reactions. **(C)** Representative microscopy images showing bright-field images of mammospheres from pLKO control and shSpy1 conditions. Total magnification of 100x. **(D)** Average number of mammospheres for pLKO control and shSpy1 conditions was determined using field of view calculation. Error bars represent standard error of the mean of three independent experiments each counted in triplicate. *$p<0.05$. **(E)** Average mammosphere diameter (µm) for pLKO control and shSpy1 conditions. Mammosphere diameter (µm) was measured using ImageJ software. Error bars represent standard error of the mean of three independent experiments each counted in triplicate. $p>0.05$. Statistical significance was assessed using a student‘s paired *t*-test.

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II. Spy1 over-expression increases the number of cells staining positive for the stemness marker CD44

To assess whether altering Spy1 levels is associated with a change in the relative stem and/or progenitor population in luminal breast cancers, MCF7 cells were manipulated to over-express or decrease Spy1 protein levels and subjected to cell surface marker analysis using the flow cytometer (Figure 6A). Cells successfully expressing pEIZ and pEIZ-Spy1 emit green fluorescence; hence infection efficiency was also monitored via flow cytometry (Figure 6B). Labelling for either CD24-PE or CD44-PE was quantified via flow cytometry analysis (representative profiles Figure 6C). Spy1 over-expression was associated with an approximately 2.5% decrease in staining for CD24 and a 10% increase in staining for CD44 (Figure 6D). These results were found to be statistically significant.

Cell populations expressing high levels of CD44 and low levels of CD24 have been shown to have stem-like properties[97,137]. Following selection with puromycin, cells exhibiting successful Spy1 knock-down (Figure 6A) were double-labelled with CD24-FITC and CD44-PE and subjected to flow cytometry analysis (representative profiles Figure 7A). Results indicate that knock-down of Spy1 is associated with an average 14% decrease in the $CD44^{high}CD24^{low}$ population (Figure 7B).

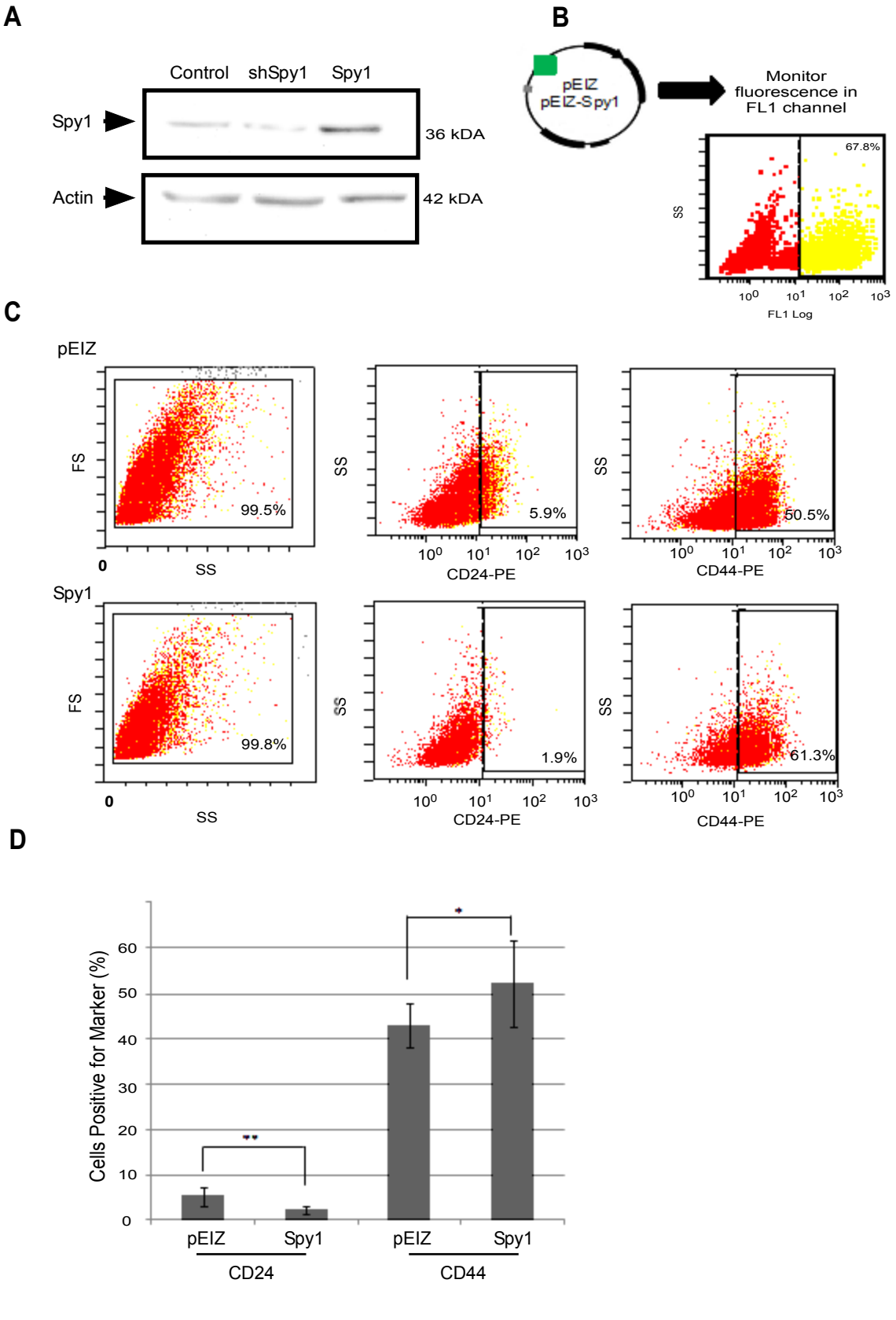
A
Control
shSpy1
Spy1
Spy1
36 kDA
Actin
42 kDA
B
pEIZ
pEIZ-Spy1
Monitor fluorescence in FL1 channel
67.8%
SS
FL1 Log
C
pEIZ
FS
99.5%
SS
5.9%
CD24-PE
SS
50.5%
CD44-PE
Spy1
FS
99.8%
SS
1.9%
CD24-PE
SS
61.3%
CD44-PE
D
Cells Positive for Marker (%)
pEIZ
Spy1
pEIZ
Spy1
CD24
CD44

Figure 6. Spy1 over-expression increases the number of cells staining positive for the stemness marker CD44.
MCF7 cells were infected with lentiviral vectors containing pEIZ as a control and pEIZ-Spy1 to over-express Spy1. **(A)** Spy1 protein levels were measured using western blot analysis. Actin served as a loading control. shSpy1 cells are utilized in Figure 7. **(B)** Successfully infected cells fluoresce green and this was monitored using the FL1 channel using flow cytometry. **(C)** Representative flow cytometry plots of either total cell populations (left panel), percentage of cells staining positive for CD24-PE (middle panel), or percentage of cells staining positive for CD44-PE (right panel) for pEIZ control and Spy1 over-expression conditions. **(D)** Average percentage of total population staining positive for CD24 and CD44 for pEIZ control and Spy1 over-expressing conditions. Error bars represent standard error of the mean of four independent experiments. *$p<0.05$ **$p<0.01$. Statistical significance was assessed using a student's paired *t*-test.

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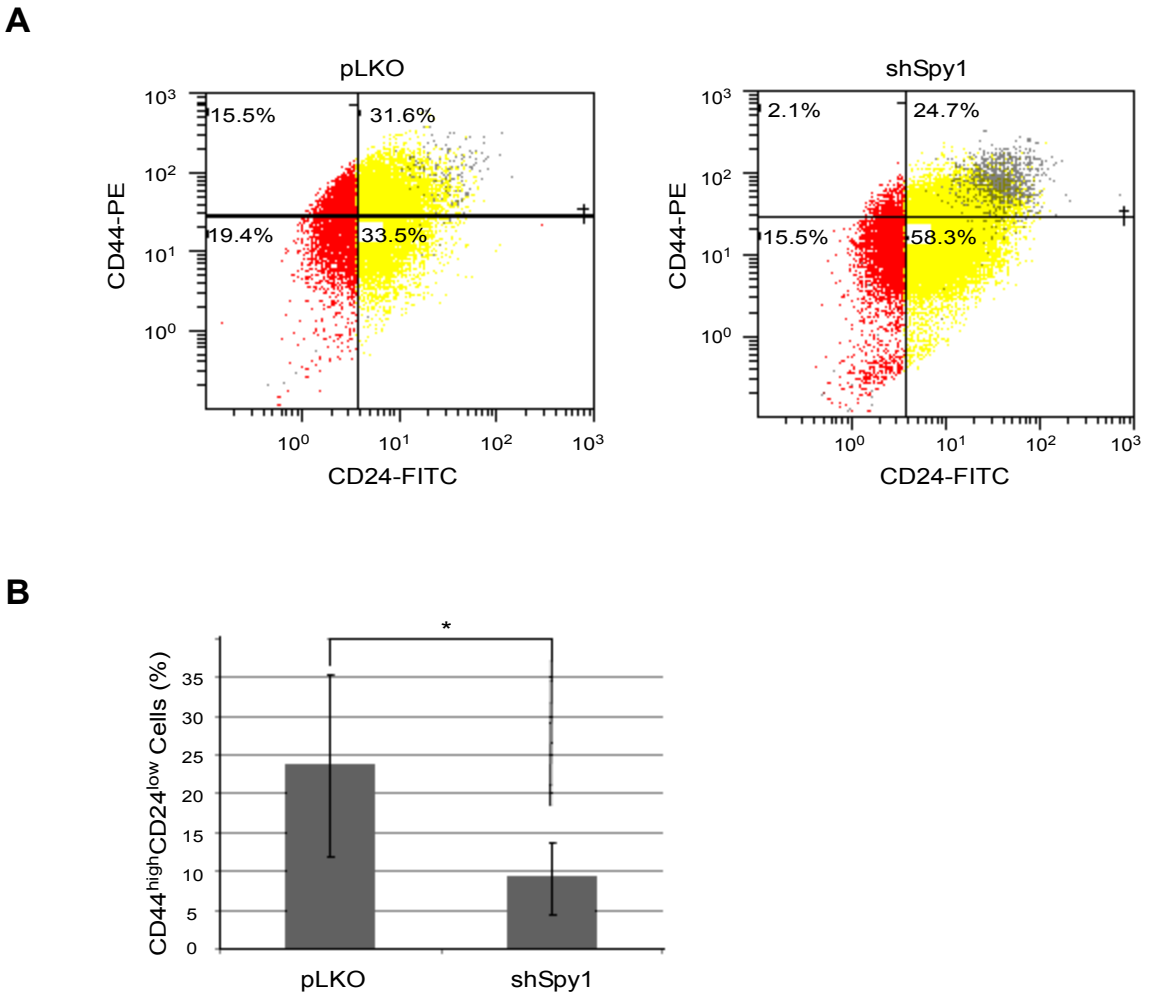

Figure 7. Spy1 knock-down decreases the stem-like $CD44^{high}CD24^{low}$ population. MCF7 cells were infected with lentivirus carrying shRNA against Spy1 (shSpy1) or a scrambled control (pLKO). **(A)** Representative flow cytometry plots showing cells double-labelled with CD44-PE (y-axis) and CD24-FITC (x-axis). Stem-like cells are found in the $CD44^{high}CD24^{low}$ population (top left quadrant). Percentages indicate the percent of cells staining positive in each fraction of the total population. **(B)** Average percentage of cells of total population staining positive in the $CD44^{high}CD24^{low}$ quadrant for pLKO control and shSpy1 conditions. Error bars represent standard error of the mean of four independent experiments. *$p<0.05$. Statistical significance was assessed using a student's paired *t*-test.

III. Spy1 knock-down decreases the ALDH positive population in triple negative and luminal breast cancer cells

As an alternate approach to cell surface marker analysis, the ALDEFLUOR® assay was utilized to examine the effect of manipulating Spy1 levels on the ALDH positive cell population in both triple negative (MDA-MB-231) and luminal (MCF7) breast cancer cell lines. ALDH positive populations are associated with various stem-like and/or progenitor characteristics[118-119]. After selection with puromycin, cells successfully expressing pLKO or pLKO-shSpy1 were incubated with the fluorescent ALDH substrate and subjected to flow cytometry analysis. MDA-MB-231 cells are known to have an ALDH positive population[8-9,142]. To control for background fluorescence, a negative control using the ALDH inhibitor DEAB was used (Figure 8A left panel). When looking at the total population, knocking-down Spy1 was associated with an average decrease of 11% in the ALDH positive population in MDA-MB-231 cells (Figure 8B). MCF7 cells are representative of the luminal subtype of breast cancer and have a relatively small percentage of ALDH positive cells compared to more aggressive subtypes of breast cancers like triple-negative breast cancers[8-9,142]. Compared to the MDA-MB-231 representative flow cytometry profiles showing percentage of cells staining positive for ALDH in pLKO and shSpy1 conditions, MCF7 cells generally had a smaller percentage of ALDH positive cells in both conditions (Figure 9A). The trend of decreased Spy1 levels and decreases in the ALDH positive population remained consistent in the MCF7 cells, and was found to be statistically significant (Figure 9B).

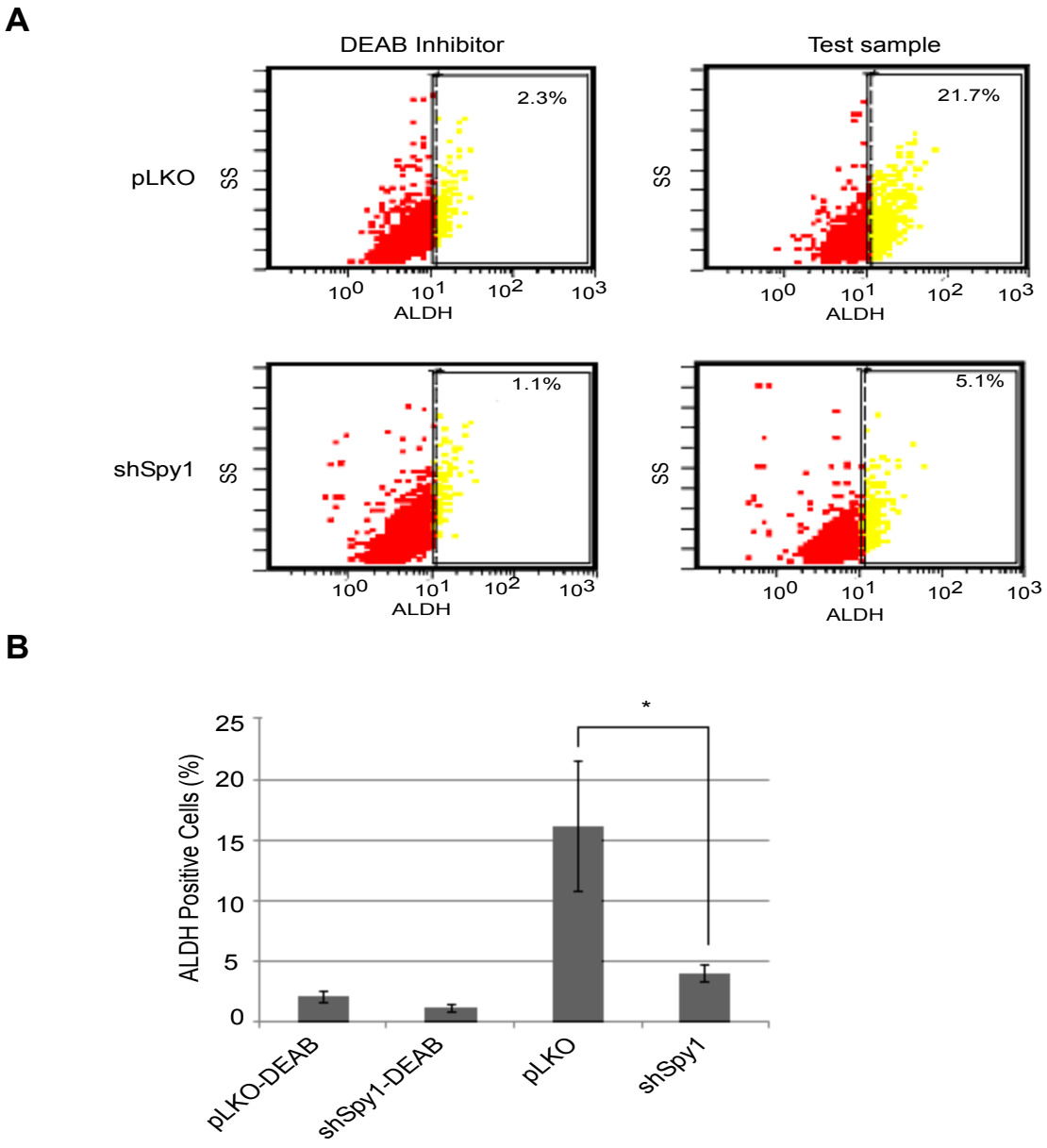

Figure 8. Spy1 knock-down decreases the ALDH positive population in triple negative breast cancer cells.
MDA-MB-231 cells were infected with lentivirus carrying shRNA against Spy1 (shSpy1) or a scrambled control (pLKO). Cells were monitored for ALDH fluorescence using the ALDEFLUOR® assay. **(A)** Representative flow cytometry plots showing percentage of ALDH positive cells for pLKO control (upper panels) and shSpy1 (lower panels) with DEAB or without the inhibitor (Test sample). Plots represent ALDH fluorescence (ALDH) vs. Side Scatter (SS). **(B)** Average percentage of cells of total population staining positive for ALDH for pLKO control and shSpy1 conditions. Error bars represent standard error of the mean of three independent experiments. *$p<0.05$. Statistical significance was assessed using a student's paired *t*-test.

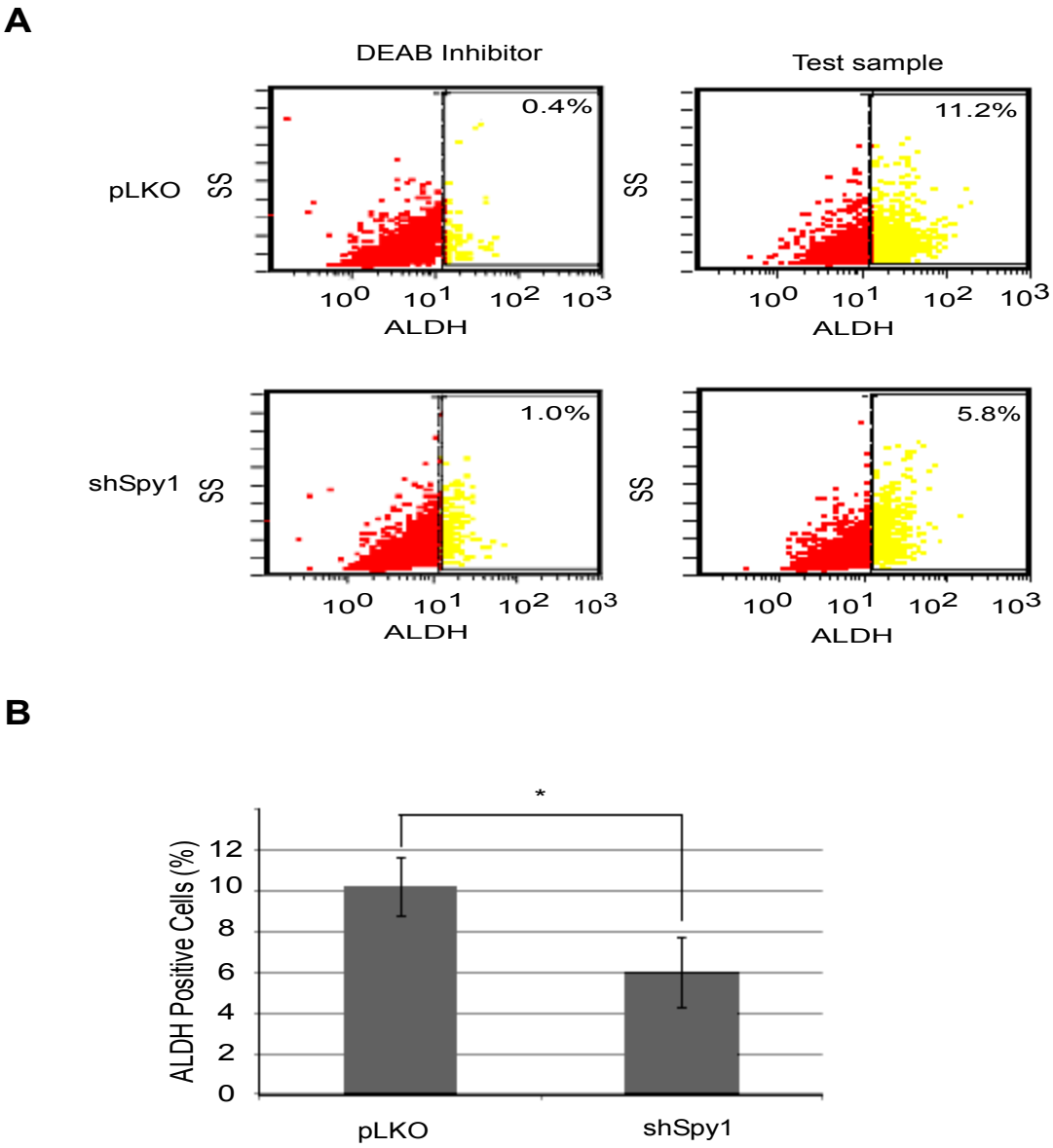

Figure 9. Spy1 knock-down decreases the ALDH positive population in MCF7 cells. MCF7 cells were infected with lentivirus carrying shRNA against Spy1 (shSpy1) or a scrambled control (pLKO). Cells were monitored for ALDH fluorescence using the ALDEFLUOR® assay. **(A)** Representative flow cytometry plots showing percentage of ALDH positive cells for pLKO control (upper panels) and shSpy1 (lower panels) with DEAB or without the inhibitor (Test sample). Plots represent ALDH fluorescence (ALDH) vs. Side Scatter (SS). **(B)** Average percentage of cells of total population staining positive for ALDH for pLKO control and shSpy1 conditions. Error bars represent standard error of the mean of three independent experiments. *$p<0.05$. Statistical significance was assessed using a student's paired *t*-test.

IV. Spy1 knock-down decreases the stem-like ALDH^{high} population in SK-BR-3 cells

SK-BR-3 cells are representative of the HER2 expressing subtype of breast cancer and were one of the original cell lines used to optimize the ALDEFLUOR® assay[143-144]. This cell line is known to contain a substantial ALDH^{high} population and is frequently used as a positive control for this assay[143-144]. ALDH^{high} cells have exhibited stem cell characteristics in normal mammary development and in breast cancer[118-119]. To assess whether altering Spy1 levels is associated with a change in the ALDH^{high} population, SK-BR-3 cells were manipulated to express decreased levels of Spy1 (shSpy1) compared to control (pLKO). Efficient knock-down of Spy1 was monitored at the protein level (Figure 10A); in addition, control and Spy1 infected cells were selected with puromycin. Flow cytometry analysis revealed a substantial percentage of the total population staining in the ALDH^{high} population (Figure 10B top right panel). Compared to control conditions, shSpy1 expressing cells exhibited a decrease in the ALDH^{high} population, as revealed by the representative flow profiles (Figure 10B bottom right panel); results revealed shSpy1 expressing cells showed an average 14% decrease in the ALDH^{high} population compared to pLKO expressing cells (Figure 10C). Overall, these results show that Spy1 knock-down decreases the stem-like ALDH^{high} population in a cell line representing the HER2+ breast cancer subtype.

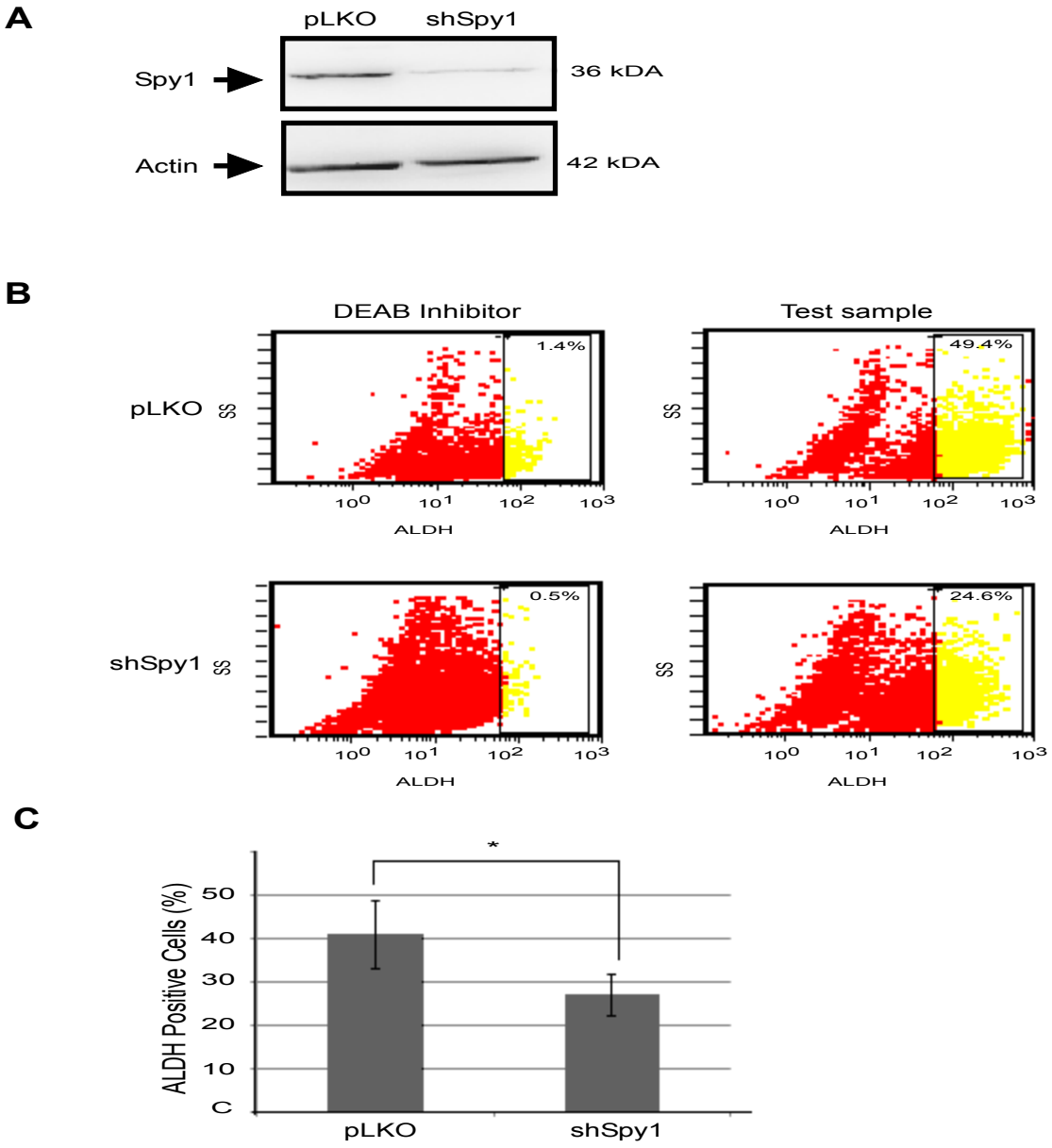

Figure 10. Spy1 knock-down decreases the stem-like ALDH^{high} population in SK-BR-3 cells.

SK-BR-3 cells were infected with lentivirus carrying shRNA against Spy1 (shSpy1) or a scrambled control (pLKO). Cells were monitored for ALDH fluorescence using the ALDEFLUOR® assay. **(A)** Spy1 protein levels were measured using western blot analysis. Actin served as a loading control. **(B)** Representative flow cytometry plots showing percentage of ALDH^{high} cells for pLKO control (upper panels) and shSpy1 (lower panels) with DEAB or without the inhibitor (Test sample). Plots represent ALDH fluorescence (ALDH) vs. Side Scatter (SS). **(C)** Average percentage of cells of total population of ALDH^{high} cells for pLKO control and shSpy1 conditions. Error bars represent standard error of the mean of three independent experiments. *p<0.05. Statistical significance was assessed using a student's paired *t*-test.

V. Knock-down of CyclinE does not cause a significant change in the ALDH positive population

To test whether decreased levels of other cell cycle regulators such as CyclinE were associated with a decrease in the ALDH positive population, MDA-MB-231s were manipulated to express reduced levels of CyclinE (shCyclinE) compared to control (pLKO). CyclinE was chosen for comparison with Spy1 because they both bind and activate CDK2 to regulate cell cycle progression. Protein levels of CyclinE were monitored through western blot analysis to ensure sufficient knock-down (Figure 11A). Representative profiles reveal similar staining patterns for both control and CyclinE knock-down conditions (Figure 11B right panel). Quantification of the ALDH positive population over three replicates revealed a very modest decrease in the ALDH positive population in the shCyclinE condition (Figure 11C); there was a large amount of variability over the three replicates and these results did not show statistical significance.

VI. Over-expression of Spy1 decreases Numb protein levels in triple negative breast cancer cells

To investigate a potential mechanism for Spy1's regulatory role in the breast cancer stem/progenitor populations, Numb protein levels were assessed in control and Spy1 over-expression conditions in MDA-MB-231 cells (Figure 12A). Numb has a role in cell differentiation as an inhibitor of Notch signalling; inhibition of the Notch pathway allows for asymmetric division to occur and subsequent differentiation[145-148]. Over-expression of Spy1 decreased Numb protein levels in MDA-MB-231 triple negative breast cancer cells (Figure 12B).

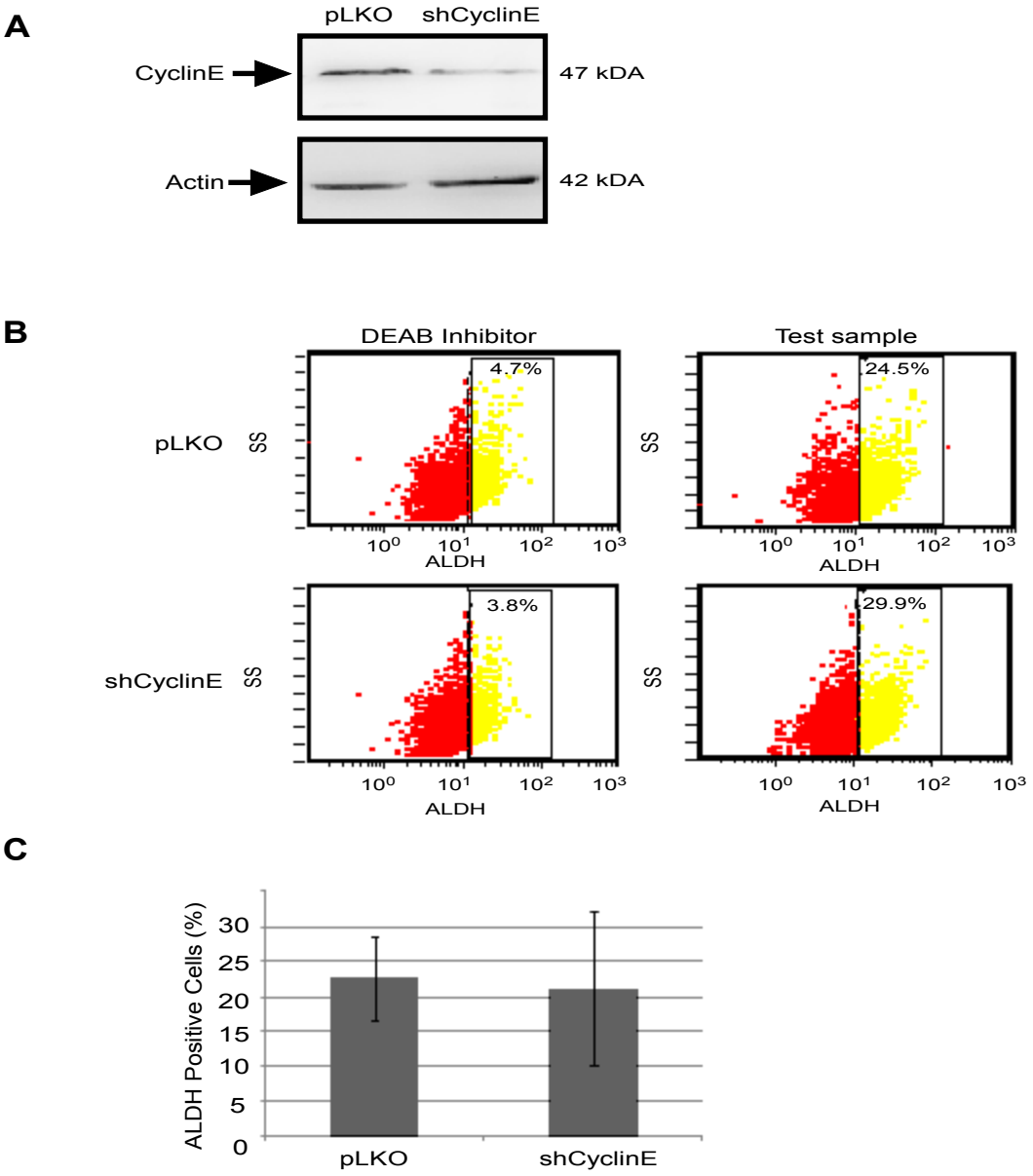

Figure 11. CyclinE knock-down does not cause a significant change in the ALDH positive population in triple negative breast cancer cells.
MDA-MB-231 cells were infected with lentivirus carrying shRNA against CyclinE (shCyclinE) or a scrambled control (pLKO). Cells were monitored for ALDH fluorescence using the ALDEFLUOR® assay. **(A)** CyclinE protein levels were measured using western blot analysis. Actin served as a loading control. **(B)** Representative flow cytometry plots showing percentage of ALDH positive cells for pLKO control (upper panels) and shCyclinE (lower panels) with DEAB (left panels) or without the inhibitor (Test sample; right panels). Plots represent ALDH fluorescence (ALDH) vs. Side Scatter (SS). **(C)** Average percentage of cells of total population staining positive for ALDH for pLKO control and shCyclinE conditions. Error bars represent standard error of the mean of three independent experiments. $p>0.05$. Statistical significance was assessed using a student‘s paired *t*-test.

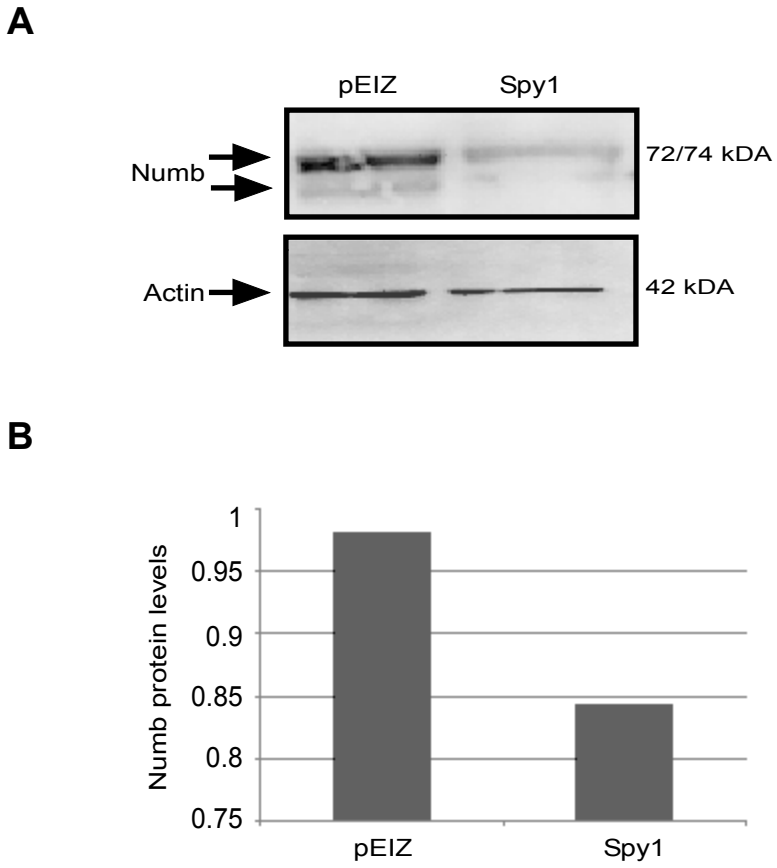

Figure 12. Over-expression of Spy1 decreases Numb protein levels in triple negative breast cancer cells.
MDA-MB-231 cells were infected with lentiviral vectors containing pEIZ as a control and pEIZ-Spy1 to over-express Spy1. **(A)** Numb protein levels were measured using western blot analysis. Actin served as a loading control. **(B)** Levels of Numb protein in pEIZ control and Spy1 over-expression conditions. Densitometry analysis depicts the average Numb protein levels of two independent experiments, each corrected to the loading control.

DISCUSSION

Breast cancer is the second leading cause of cancer deaths in Canadian women, claiming the lives of approximately 14 women each day[82]. Although advances in earlier detection and treatment have improved patient outcomes, further understanding the complex heterogeneity of this disease is critical in improving patient response to therapy and in the prevention of relapse. Dissecting the roles of different sub-populations found in breast cancer tumours has revealed aggressive populations with stem cell characteristics as these cells have been shown to recapitulate tumours in transplantation experiments[97], display increased invasiveness properties[138], and exhibit many phenotypic and functional characteristics similar to normal mammary stem cells[24-25,32,95]. Understanding how these populations are regulated is necessary for the development of targeted approaches in a clinical setting. This work reveals that the atypical cell cycle regulator Spy1 is involved in regulating the breast cancer stem and/or progenitor populations found in various breast cancer subtypes.

To investigate if Spy1 regulates breast cancer stem and/or progenitor populations, Spy1 levels were manipulated in a triple negative breast cancer cell line and subsequently subjected to *in vitro* mammosphere assays. MDA-MB-231 cells are known to contain a sub-population of breast cancer stem cells and endogenously express high levels of Spy1[79,142]. Over-expressing Spy1 in the cell line revealed an increase in the number of mammospheres formed. This is consistent with previous findings in the brain showing Spy1 over-expression increases neurosphere formation. After 7 days in culture, mammospheres were highly fluorescent, indicating successful lentiviral infection and high Spy1 expression within the mammosphere structures. To validate these findings, we

also knocked-down Spy1 expression in MDA-MB-231 cells. A significant decrease in the number of mammospheres formed was revealed, suggesting a role for Spy1 in the sub-population of cells involved in mammosphere formation. It has been established that only cells with stem cell characteristics, mainly the ability to self-renew and give rise to differentiated cells, are able to form mammospheres in culture[106-107]. Transcriptional profiling of mammospheres demonstrated differential gene expression profiles compared to cells in adherent cultures. Up-regulation of genes required in homing (e.g., CXCR4), maintaining cells in an undifferentiated state (e.g., IL6), and regulation of self-renewal (e.g., Wnt pathway) were found in mammopsheres[106]. Future experiments will look at potential transcriptional changes within mammospheres when Spy1 is over-expressed.

Previous work has revealed a correlation between mammosphere size and the ability of mammosphere cells to form tumours in immunocompromised mice, suggesting that larger mammospheres contain more stem-like cells with the ability to form tumours when injected into cleared mammary fat pads[149]. Spy1 knock-down did not significantly decrease mammosphere size compared to control. This may be due to the observation that MDA-MB-231 cells normally exhibit variation in mammosphere structure, as opposed to neurospheres that form uniform spherical multicellular structures, making it challenging to detect small differences in mammosphere size[40,149]. To directly assess whether Spy1 is affecting stem cell self-renewal, a FACS experiment is required, which is an important future direction for this project. Cells derived from mammospheres that are over-expressing Spy1 can be sorted based on a successfully incorporated green fluorescent tag into single cell suspension and clonal analysis can be performed. If single cells over-

expressing Spy1 show an enhanced ability to form multicellular structures *in vitro*, it suggests a role for Spy1 in regulating self-renewal parameters.

To further elucidate a potential role for Spy1 in the breast cancer stem cell and/or progenitor populations, cell surface marker analysis was completed using flow cytometry. Previous work has revealed a specific cell surface marker phenotype for breast cancer cells enriched with stem-like characteristics; cells marked with a $CD44^{+/high}CD24^{-/low}$ phenotype show enhanced mammosphere forming ability, increased invasive properties, and the ability to recapitulate tumours when transplanted into the cleared fat pads of immunocompromised mice[97,137-138]. Spy1 levels were manipulated in MCF7 cells, representing the luminal A breast cancer subtype[8-9]. MCF7 cells represent a less clinically aggressive subtype of breast cancer and have relatively low levels of Spy1 compared to MDA-MB-231s[8-9,87]. Cells over-expressing the control pEIZ or Spy1 were tagged with a fluorescent green marker by lentiviral infection to monitor successful infection over the course of multiple repeats. To avoid overlap with expression of cell surface marker antibodies conjugated to green tags, samples were labelled separately with either CD24-PE or CD44-PE. Spy1 over-expression significantly increased levels of CD44 and significantly decreased the levels of CD24 compared to control in MCF7 cells. These findings suggest a role for Spy1 in regulating the expansion of the CD44+ sub-population. CD44 plays many important roles in CSCs, aiding in motility, the maintenance of stemness through ligand-receptor interactions, and drug resistance[99,150-152]. While CD44 and CD24 are considered standard cell surface markers for identification of stemness, there is a distinction to be made for separating $CD44^{+}CD24^{-}$ from $CD44^{high}CD24^{low}$ [139]. Both approaches show enrichment for the desired sub-population,

however cells expressing a $CD44^{high}CD24^{low}$ are thought to contain a higher percentage of breast cancer stem cells[137,139,153-154]. Thus, to investigate whether Spy1 alters the balance of the $CD44^{high}CD24^{low}$ sub-population, Spy1 levels were subsequently knocked-down, cells were double-labelled with CD24-FITC and CD44-PE and subjected to flow cytometry analysis. Results revealed that Spy1 knock-down consistently decreased the percentage of $CD44^{high}CD24^{low}$ cells over three repeats, demonstrating a statistically significant change. This result suggests a role for Spy1 in altering the balance between breast cancer stem cell enriched versus non-enriched sub-populations. These finding are consistent with the mammosphere data, further strengthening the support for Spy1's potential role in regulating the breast cancer stem/progenitor populations. Sorting cells expressing a $CD44^{high}CD24^{low}$ phenotype and subsequently subjecting the isolated population to clonality assays and *in vivo* transplantation assays will verify that this sub-population is enriched for breast cancer stem cells[97,137], further validating this experimental model. Manipulating levels of Spy1 within $CD44^{high}CD24^{low}$ expressing cells will determine the essentiality of Spy1 in this population.

As an alternate method to assess Spy1's role in regulating breast cancer stem/ progenitor populations, the Aldefluor® Assay was performed; Spy1 levels were manipulated in MDA-MB-231 and MCF7 cells and the $ALDH^+$ population was monitored using flow cytometry. It has been previously suggested that cells positive for ALDH have enhanced stem cell characteristics, both in normal mammary development and in breast cancer[10,118-119]. Approximately 16% of MDA-MB-231 control cells were positive for ALDH (n = 4), consistent with findings in the literature that triple negative breast cancers have a known $ALDH^+$ population[118]. Compared to control, Spy1 knock-

down decreased the $ALDH^+$ population to less than 5% on average. This finding, in conjunction with the mammosphere data using MDA-MB-231s, supports a role for Spy1 in breast cancer stem/progenitor populations. MCF7 cells were also subjected to the Aldefluor® Assay and control cells had an average lower percentage of cells staining positive for ALDH compared to MDA-MB-231s. Compared to control, Spy1 knock-down decreased the $ALDH^+$ population by an average 4%, although the change was less substantial compared to the change seen in MDA-MB-231s. This is expected, as MCF7 cells endogenously have lower levels of Spy1 and are known to contain a comparatively smaller fraction of breast cancer stem cells[142]. These results are consistent with the decrease in the amount of cells staining positive for $CD44^{high}CD24^{low}$ in MCF7 cells with knock-down of Spy1. Overall these results indicate that cells expressing lower levels of Spy1 show a significant decrease in the $ALDH^+$ sub-population, known to contain cancer cells with stem cell characteristics. This effect held true across two different breast cancer subtypes.

Similar to cell surface marker expression, the literature shows variation in whether researchers use an $ALDH^+$ phenotype versus an $ALDH^{high}$ phenotype. Although both show enrichment for the breast cancer stem cell sub-population, cells expressing high levels of ALDH are thought to contain a higher percentage of breast cancer stem cells[10,118]. The SK-BR-3 cell line was one of the original cell lines used to optimize the Aldefluor® Assay for breast cancer samples[143-144]. SK-BR-3 cells have a known population of $ALDH^{high}$ cells, and can be used as a positive control[143-144]. SK-BR-3 also cells have high Spy1 levels, similar to that of MDA-MB-231s. Thus, to investigate whether Spy1 alters the balance of the $ALDH^{high}$ sub-population, Spy1 levels were

knocked-down in SK-BR-3 cells and the effect on the $ALDH^{high}$ sub-population was assessed using flow cytometry analysis. Knock-down of Spy1 resulted in a significant decrease in $ALDH^{high}$ cells, with an average 10% decrease compared to control cells. These results further elucidate a role for Spy1 in regulating the stem-like population found within the $ALDH^{high}$ fraction in SK-BR-3 breast cancer cells.

In general, characterizing the stem cell population within breast cancers has been challenging due to the lack of definitive markers compared to other cancers. For example, brain tumour-initiating cells can be isolated based on CD133 expression; cells expressing CD133 can be magnetically sorted and subsequently be subjected to *in vitro* and *in vivo* analysis[80]. This is in contrast to high/low expression in which magnetic sorting is not feasible; instead sub-populations need to be carefully gated and sorted based on fluorescence for precise isolation. When looking at the effect of manipulating Spy1 on the breast cancer stem/progenitor populations, it was necessary to take multiple methodological approaches in order to verify the results. Thus, manipulated cell lines were subjected to mammosphere assays, cell surface marker analysis and the Aldefluor® Assay; results were therefore corroborated through a variety of different assays, strengthening support for a potential role for Spy1 in regulating the stem/progenitor populations in breast cancers. To directly assess whether Spy1 is affecting the breast cancer stem/progenitor population, it will be necessary to sort the populations and perform analysis on the isolated population. For example, the $ALDH^{high}$ fraction can be sorted, Spy1 levels can subsequently be knocked-down, and the effect on mammosphere forming ability can be assessed *in vitro*, or subjected to *in vivo* transplantation experiments. These experiments are part of important future directions for this project.

Breast cancer is an extremely heterogeneous disease, at both the histological and molecular levels. The disease is classified into different subtypes, in which a variety of different breast cancer cell lines exist as representative models[8-9,139-140]. Three different breast cancer cell lines were utilized in this study to examine whether Spy1 was an important regulator across the most prevalent of these subtypes. It was found that manipulating Spy1 had a significant effect on the relative stem/progenitor populations in cell lines representing triple negative, luminal, and HER2 over-expressing breast cancers. Manipulating Spy1 in MDA-MB-231s and SK-BR-3 cells revealed the most substantial differences when comparing control to experimental conditions. These findings are consistent with the observation that both of these cell lines have comparatively high levels of Spy1[79]. Statistically significant differences were also seen in MCF7 cells when comparing control to experimental conditions, although the differences were more subtle compared to the other cell lines. These results are consistent with the observation that MCF7s have comparatively low levels of Spy1. Interestingly, MDA-MB-231s, which have high levels of Spy1, are clinically very aggressive and typically respond poorly to conventional therapies[94]. The poor clinical response may be reflective of expansion of the breast cancer stem population with enhanced protective mechanisms that are both driving tumourigenesis and impeding therapeutic response.

Spy1 is an attractive candidate for regulating the growth of the aggressive breast cancer stem cell population. It has been established in the brain that Spy1 levels are elevated in clonally derived neurospheres and decrease during stages of differentiation[80]. In addition, increased Spy1 levels serve as a block to differentiation and increase the number and life-span of neural stem/progenitors in culture[80]. For functional

differentiation to occur Cdk2 activity must decrease and p27 protein levels must increase[155-156]. Spy1 can activate Cdks in an atypical manner and Spy1-Cdk2 complexes are less sensitive to inhibition by certain CKIs[74]. In addition, Spy1 can bind and promote the degradation of p27[72,75], which may allow for expansion of the stem cell population when normally cell cycle progression would be inhibited. Spy1 knock-down, but not cyclinE knock-down, decreased the $ALDH^+$ population in triple negative breast cancer cells, suggesting a unique role for Spy1 in regulating the stem-like population. Perhaps these findings are due to the atypical nature of Cdk activation and ability to promote the degradation of p27 characteristic of Spy1 that allows for this unique role.

A hallmark characteristic of normal stem cells is the ability to shift between symmetric and asymmetric division[23]. Cancer stem cells shift the balance to favour symmetric division, as it allows for the rapid expansion of the aggressive stem cell population in tumours[23]. In BTICs, Spy1 demonstrated an important role in maintaining symmetric division, as revealed through Numb distribution assays[80]. Numb's primary role in cell differentiation is as an inhibitor of Notch signalling; inhibition of the Notch pathway allows for asymmetric division to occur and subsequent differentiation[145-147]. When the protein Numb is distributed unevenly throughout a cell, this promotes asymmetric division because it influences the response of the daughter cells to Notch signaling, yielding two distinct cell fates[145]. Numb can be repressed at a translational level by Musashi-1 (Msi1); this allows for activation of Notch signalling in the absence of the inhibitor Numb (Figure 13)[157]. Interestingly, Spy1-CDK signalling has been shown to activate Musashi-1 (Msi1)[157]. Spy1-CDK activation of Msi1 may serve as a mechanism for Spy1's potential regulatory roles in the breast cancer stem/progenitor

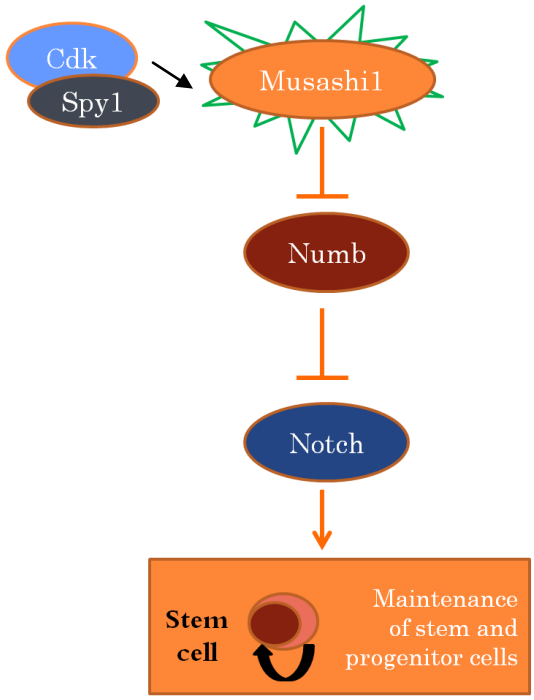

Figure 13: Potential mechanism for Spy1's regulatory role in the breast cancer stem and/or progenitor populations

Spy1-CDK signalling has been shown to activate Musashi-1. Numb can be repressed at a translational level by Musashi-1[157]; this allows for activation of Notch signalling in the absence of the inhibitor Numb[145-147]. Notch signalling is an important pathway regulating self-renewal decisions and thus contributes to the maintenance of stem and progenitor populations[43, 51-53].

populations. Preliminary data reveal that over-expression of Spy1 in the heterogeneous population of triple negative breast cancer cells correlate with a decrease in Numb protein levels. The connection between Spy1 and Numb will be further probed in cell sorted populations to investigate the potential mechanism for regulation.

This is the first study to investigate the potential role of Spy1 in stemness properties in breast cancer. In summary, over-expression of Spy1 increases the mammosphere forming ability of breast cancer stem/progenitor cells and increases overall levels of the stemness marker CD44; similarly, knock-down of Spy1 decreases the number of mammospheres formed, and decreases the $CD44^{high}CD24^{low}$, $ALDH^{+}$, and $ALDH^{high}$ sub-populations (Table 4). Collectively, these findings provide strong support that Spy1 plays a regulatory role in breast cancer stem and/or progenitor populations. The cancer stem cell model has important clinical implications and understanding the different sub-populations driving tumourigenesis is crucial to the development of targeted clinical approaches (Figure 14)[30,32,95]. Dissecting the key regulators of the most aggressive breast cancer stem and/or progenitor populations will aid target-specific approaches, ultimately improving patient outcome. The findings in this study may have clinical implications toward targeted approaches in the treatment of breast cancer.

Table 4: Effect of Spy1 on the relative stem cell population within various breast cancer cell lines

Breast cancer cell line	**Relative Spy1 levels[79]**	**Mammosphere assay**	**$CD44^{high}CD24^{low}$ cell surface marker analysis**	**ALDEFLUOR ASSAY®**
MCF7 Luminal A	low	-	↑ Spy1= ↑ CD44 staining ↓ Spy1 = ↓ $CD44^{high}CD24^{low}$ population	↓ Spy1= ↓ $ALDH^{+}$ population
SK-BR-3 HER2 Positive	high	-	-	↓ Spy1= ↓ $ALDH^{+}$ population
MDA-MB-231 Claudin-Low	high	↑ Spy1= ↑ number of mammospheres ↓ Spy1= ↓ number of mammospheres	-	↓ Spy1= ↓ $ALDH^{+}$ population ↓ CyclinE = no change $ALDH^{+}$ population

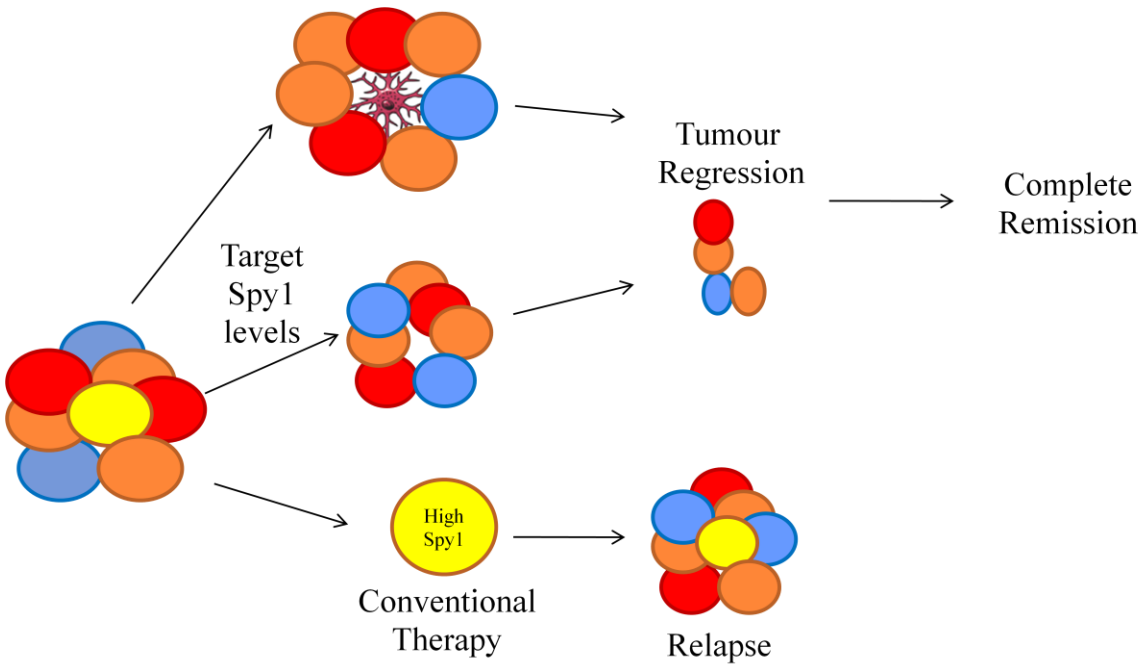

Figure 14: The significance of cancer stem cell directed targeting strategies

Cancer stem cells (yellow), compared to more differentiated cells (blue, orange, red), have enhanced protective mechanisms that make them resistant to conventional therapies and may be responsible for relapse[30,32,95]. Designing treatment options to target this aggressive population by elimination or coaxing them to a more differentiated state may aid in complete remission after treatment.

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