SIDE POPULATION IN HUMAN LUNG CANCER CELL LINES AND TUMOURS IS ENRICHED WITH STEM-LIKE CANCER CELLS

by

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Abstract

An emerging concept in cancer initiation and development is that cells with characteristics of the stem cell phenotype drive tumour growth and progression. Accumulating evidence reveals that solid tumours such as brain and breast cancer contain primitive cancer stem cells that have high repopulation capacity. Recent studies have demonstrated that adult stem cells can be identified by a side population (SP) phenotype. The work in this thesis was the first in investigating the existence of cancer stem cells in human lung cancer. This study used flow cytometry and the Hoechst 33342 dye efflux assay to isolate and characterise SP cells from various human lung cancer cell lines. In addition, the existence of SP cells in lung cancer tissues obtained from surgical resection was also investigated. Results indicated that all six human lung cancer cell lines contained an SP that could be reliably detected under the experimental conditions used. Evidence was found for asymmetric division by the SP to generate a population resembling the original unsorted population. SP cells and non-SP cells were characterised at the molecular level to compare mRNA expression between the two populations. SP cells from each cell line displayed elevated expression of ATP-Binding Cassette (ABC) transporters associated with multi-drug resistance. In particular, expression of ABCG2 (BRCP1) defines the SP phenotype. Human telomerase reverse transcriptase (hTERT) expression was higher in the SP cells, suggesting this fraction may represent a reservoir with high proliferative potential for generating cancer cells. mRNA levels of MCM7, a member of the mini-chromosome maintenance (MCM) family of proteins, a critical component of the DNA replication complex, was lower in SP cells suggesting that a majority of the cells from the SP fraction were in G0 of the cell cycle.
Levels of mRNA expression of BMI-1, a pathway associated with stem cell self-renewal, were higher in the SP cells compared to the non-SP cells in four of the six cell lines. The Notch-1 pathway was another self-renewal pathway that had increased mRNA expression in five of six cell lines. Functional characterisation of the SP and non-SP were investigated in both *in vitro* and *in vivo*. The Matrigel™ invasion assay demonstrated SP cells as having higher potential for invasiveness than non-SP cells, suggesting there exist a population of stem-like cells within a lung tumour that is involved in the initiation of invasion and metastasis. Non-obese diabetic/severe combined immunodeficiency (NOD/SCID) xenograft transplant experiments showed that SP cells were more tumourigenic compared to non-SP cells. Using a cell viability assay, SP cells were determined to exhibit higher resistance to drugs used to treat lung cancer, some of which are substrates for ABC transporters. Staining patterns of other putative stem cell-related surface markers such as CD24, CD34, CD44 and nestin were also examined and compared between the SP and non-SP. Using the Hoechst efflux assay, SP cells were also isolated from patient tissues, which contained a low but persistent percentage (0.025 – 1.08) of SP cells. Taken together, these studies suggested that SP is an enriched source of tumour re-initiating cells with stem cell properties and may be an important target for effective therapy and a useful tool to investigate the tumourigenic process.
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List of Abbreviations

ABC, ATP-binding cassette
APC, allophycocyanin
BCRP, breast cancer resistance protein
BMI, B lymphoma Mo-MLV insertion region
CT, critical threshold
DEPC, diethylpyrocarbonate
DMEM, Dulbecco’s modified Eagle’s medium
DMSO, dimethyl sulphoxide
EDTA, ethylene-diamine-tetra-acetic acid
EMT, epithelial to mesenchymal transition
FACS, fluorescence activated cell sorting
FBS, foetal bovine serum
FCS, foetal calf serum
GAPDH, glyceraldehyde-3-phosphate dehydrogenase
HBSS, Hank’s balanced salt solution
HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFN, Hank’s balanced salt solution + 2% FCS + 0.05% NaN₃
hTERT, human telomerase reverse transcriptase
IC₅₀, half maximal inhibitory concentration
MCM, mini-chromosomal maintenance
MDR, multidrug-resistance
MEM, minimum essential medium
MRP, multidrug-resistance-associated protein
MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NCBI, National Center for Biotechnology Information
NOD-SCID, non obese diabetic severe combined immunodeficient
Non-SP, non side population
PE, phycoerythrin
PI, propidium iodide
RT-PCR, real-time polymerase chain reaction
Shh, Sonic hedgehog
SP, side population
UV, ultraviolet
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1.0 INTRODUCTION

1.1 Lung Cancer
Lung cancer is the most common cause of cancer death in the world today [1]. Currently, lung cancer survival is poor with only 15% of patients surviving 5 years after diagnosis. While new chemotherapy agents and radiotherapy have improved survival and quality of life of patients, the overall impact in the last decade has been mainly on diminishing of the severity of the disease without removing the cause rather than a reduction in mortality. Lung cancer is classified into two major groups according to the histological appearance of the malignant cells: small cell lung carcinoma (SCLC), accounting for approximately 20-25% of lung cancer, and non-small cell lung carcinoma (NSCLC) which accounts for almost all remaining cases of primary lung cancers. Non-small cell lung carcinomas can be further subdivided into 3 subgroups: lung adenocarcinoma, squamous cell carcinoma and large cell lung carcinoma.

Squamous cell carcinomas arise from the epithelial cells that line the surfaces of the centrally located large and medium sized bronchi, and have been demonstrated to develop in a serial step-by-step pathologic sequence from squamous metaplasia, to increasingly severe grades of dysplasia, to in situ carcinoma resulting in advent of submucosal invasion and metastatic disease. This occurs on a background of widespread increase in bronchial epithelial cell proliferation likely as a result of tobacco smoke induced release of growth factors such as bombesin [2]. The changes associated with adenocarcinoma are however more vague and controversial. Peripheral adenocarcinoma arises in the bronchoalveolar junction. The mucous cells and the precursor cells of
bronchioles and alveoli (Clara cells and type II alveolar pneumocytes) in this location are distinct from those of the large and medium bronchi. However, these epithelial cells also appear to follow a multistage model in cancer.

1.2 Cancer stem cells
An emerging important theme in cancer research in the past few years is the concept that the growth and progression of a tumour is fuelled and sustained by a rare, biologically distinct population of cells (termed “cancer stem cells”) within the tumour. These cells display stem-like properties, in particular, the ability for self-renewal and differentiation. The concept of cancer stem cells was first proposed by pathologists Rudolf Virchow and Julius Cohnheim about 150 years ago. They observed histological similarities between the embryonic tissue and certain types of cancers such as teratocarcinomas with respect to their enormous capacity for both proliferation and differentiation [3, 4]. A study by Jacob Furth and Morton Kahn in 1937 discovered that a single leukaemic cell was able to transfer the systemic disease when transplanted by inoculation into a mouse, and thus established the first quantitative assay to determine the frequency of malignant cells within the haematopoietic tumour [5]. In the 1960s, Robert Bruce and Hugo van der Gaag developed a quantitative method to measure the number of murine lymphoma cells capable of proliferating in vivo using a spleen colony-forming assay. They showed that only a small subset of primary tissue was able to proliferate in vivo [6]. Another study conducted in 1977 by Anne Hamburger and Sydney Salmon reported that only 1 in 1,000 to 1 in 5,000 cancer cells formed colonies in soft agar assay and thus established that not every cancerous cell is capable of tumour initiation [7]. Taken together, these studies
suggested that not every cell is able to proliferate to form a colony *in vitro* or give rise to a tumour *in vivo*.

These studies eventually led to the identification of the tumour-initiating cell, or the “cancer stem cell,” in 1997 by Dominique Bonnet and John Dick [8]. Using a characteristic marker signature, they prospectively purified a small subset of leukaemic cells in acute myeloid leukaemia that was able to establish leukaemia in an immunodeficient mouse model. This study presented direct evidence for the hierarchical model of cancer that the tumour consisted of a population of cancerous cells with differing degrees of tumour-initiating capability. It provided the framework for enriching these cancer stem cells and challenged the basis of the stochastic model, which viewed that each individual cell within entire tumour population possessed the ability to generate new tumours.

The research on cancer stem cells in haematopoietic malignancy has been carried over to solid tumours in recent years. Evidence is accumulating that solid tumours such as brain and breast cancer also contain a minor population of cancer stem cells that have high repopulation capacity. Muhammad Al-Hajj and colleagues identified and prospectively isolated a small subset of cells in human breast tumours that had the ability to initiate phenotypically heterogeneous tumours *in vivo* using the cell surface marker profile CD44+/CD24− [9]. In another study, Sheila Singh and colleagues isolated a small fraction of human brain tumour cells expressing the neural stem cell marker CD133 that could differentiate in culture into tumour cells resembling the original patient’s tumour [10].
These studies suggested that only a small population of cells within the tumour have the potential to drive the growth of these tumours. These cells displayed stem-like properties, in particular, the ability to self-renew and the capacity to establish tumour heterogeneity. This is consistent with the cancer stem cell model in that most cancer cells have only limited proliferative potential and only a small distinct subset of cancer cells can consistently form new tumours upon transplantation, as opposed to the model that all cancer cells have the potential to establish new tumours [11] (Figure 1).

**Figure 1. Two models of solid cancer cells.**

(a) Solid tumours consist of different phenotypes of cancer cells that all have the potential to proliferate extensively and form tumours.

(b) Only the cancer stem cells (CSC) have the ability to proliferate extensively and form new tumours while the remaining cells cannot.

![Figure 1](image-url)

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The existence of cancer stem cells within lung cancer is highly probable. *In vitro* data from the Hamburger and Salmon study also showed that only 1 in 1000-5000 cancer cells in lung cancer form colonies in soft agar assay, which indicates that not every lung cancer
cell is capable of tumour initiation [7]. Recent data from a mouse model reinforce the notion that lung adenocarcinoma arises from stem cells in the terminal bronchioles [12]. Hence, it is likely that human lung cancers have rare, stem-like cancer-initiating cells within the tumour.

1.2.1 Definition of cancer stem cells

Normal stem cells exist throughout the body as adult stem cells, replenishing tissue through the tightly regulated process of renewing cells. These cells are very rare and have extraordinary proliferative potential, but are usually quiescent in their niches. They are defined by three fundamental properties [13, 14]:

1) Stem cells have the ability of self-renewal to maintain the original stem cell population. Self-renewal is a cell division in which one or both new daughter cells are formed with equal proliferative and differentiation potential as the initial parent cell.

2) Stem cells must be able to differentiate and give rise to heterogeneous progeny cells to replace the mature cells that turn over in adult tissues.

3) Stem cells are strictly regulated and have the ability to control differentiation and self-renewal according to the requirements of the surrounding tissue.

These defining properties of the stem cell are demonstrated by its ability to reconstitute an entire organ, as illustrated by a single haematopoietic stem cell having the ability to regenerate the whole blood system of an irradiated mouse [15].
Cancer stem cells are considered as stem cell-like cells in a tumour. There exists a small subset of cells within the heterogeneous population of cancerous cells in a patient tumour that have similar normal stem cell properties of differentiation and self-renewal. However, it is more difficult to define the cancer stem cell compared to its normal counterpart. One can define cancer stem cells as a prospectively purified population within the tumour that has much higher tumourigenic ability compared to the remaining bulk population of the tumour. Alternatively, a stricter definition of cancer stem cells would require a single, isolated cancer stem cell to reconstitute an entire new tumour in a recipient animal that is identical to the original patient tumour. A single cancer stem cell must also have the ability to be serially xenotransplanted indefinitely. But to expect that the single cancer cell can establish an entire new tumour similar to the original patient tumour in a foreign host environment will be extremely demanding and difficult to prove. Furthermore, when injecting the single cancer stem cell into the recipient animal, the area of inoculation chosen is only similar to the original human tissue and lacks essential supporting cells (i.e. stromal cells) for tumour establishment. The definition of the cancer stem cell is not as apparent as defining the normal adult stem cell.

With these complications, one must consider the intermediate between both ends of the definition as a compromise when establishing the criteria that defines the putative cancer stem cell. Instead, the cancer stem cell can be defined as a prospectively purified small subset population that is enriched in tumour initiating cells using \textit{in vivo} tumourigenicity experiments. This small population must also possess certain intrinsic properties similar to normal adult stem cells, such as differentiation and self-renewal. Rather than
attempting to isolate the single putative cancer stem cell, these conditions allow a
candidate subset of cells enriched with tumour initiating potential to be defined as a
population of stem-like cancer cells within the tumour [16].

1.2.2 Origin of cancer stem cells
The cellular origin of cancer stem cells is unknown. There are several possible causes
that might give rise to cancer stem cells (Figure 2). The normal adult stem cells and the
early stem progenitors may have undergone specific mutations that affect suppression of
their proliferative and expansive properties, transforming them into malignant stem cells.
Normally, these cells have much longer life-span compared to the differentiated cells,
thereby allowing more opportunities for mutations to accumulate over time. However,
cancer stem cells may also be derived from the differentiated cells through the process of
dedifferentiation. The differentiated somatic cells may mutate and become less
differentiated, dividing rapidly and acting more like the normal stem cell. It is difficult to
definitively determine the numerous factors that can cause the emergence of cancer stem
cells, yet the fact remains that these cells are malignant cells displaying stem-like cell
properties [17, 18]. Hence, irrespective of the origin of cancer stem cells, this rare
population displays stem cell properties, notably, the ability to self-renew and to
differentiate into a functional hierarchy of tumourigenic and non-tumourigenic cells.
Figure 2. Origins of cancer stem cells.

Cancer stem cells might appear after mutations in stem cells or progenitor cells cause them to become unregulated. These cells may arise from differentiated cells through the mutations that cause dedifferentiation. Environmental factors from the host may also trigger the formation of tumours from stem cells.

1.2.3 Common properties between normal and cancer stem cells

There are many parallels between normal stem cells and cancer stem cells. Normal and cancer stem cells share similar surface-marker phenotypes, as evident in leukaemic stem cells and normal haematopoietic stem cells [8, 19]. This supports the concept that cancer stem cells may arise from normal stem cells. Furthermore, similar signalling pathways may regulate self-renewal and differentiation in both stem cells and cancer stem cells.

Signalling pathways classically associated with cancer are shown to also regulate normal stem cell development [11]. The Notch, Sonic hedgehog (Shh) and Wnt signalling
pathways are all important contributors to self-renewal mechanisms in normal stem cell development in the haematopoietic and nervous systems [20-22]. Notch-1 is known to be involved in inhibitor of differentiation in stem cells. When a mutation occurs that disrupts the regulation of these pathways, it can contribute to oncogenesis, as shown in various human tumours including colon carcinoma [23], medulloblastoma [24] and leukemia [25].

Normal stem cells are known to have protective properties such as resistance to drugs and toxins through increased expression of several ATP-binding cassette (ABC) transporters, effective DNA repair capacity and resistance to apoptosis [26, 27]. It is possible that cancer stem cells might also have these resistance mechanisms. The Goldie-Coldman Hypothesis proposed that a small percentage of cells in a population harbour intrinsic mutations that results in resistance to chemotherapeutic agents [28].

1.2.4 Implications of solid cancer stem cells

An important implication of solid cancer stem cells is the occurrence of tumour re-establishing after regression from initial therapy. Existing conventional therapies mainly target cells that are rapidly undergoing cell division and are not as effective towards cells that are slowly dividing. The solid cancer stem cells may therefore be less sensitive towards these therapies and thus remain viable after treatment and re-establish the tumour. Furthermore, the cancer stem cells may have intrinsic protective properties and be more resistant to chemotherapy. If therapies are developed to target cancer stem cells, rather than the bulk of the tumour, the tumours will be deprived of the cancer stem cells and be unable to maintain themselves or grow. Cancer stem cell-directed therapies may
eventually lead to better treatment outcomes even if they do not shrink tumours initially [11] (Figure 3).

Currently, most tumours are treated as though all cancerous cells have unlimited proliferative potential and have the ability to metastasize. However, studies have now shown that tumours consist of a heterogeneous population and that not all cancerous cells have equal ability to re-initiate new tumours. Some cancer cells at sites distant from the primary tumour are not able to form metastatic disease [7, 14]. Rather, the formation of new tumours may only occur due to the invasion of the cancer stem cell to new sites leading to metastatic disease. Therefore it may be essential that therapies target and kill the cancer stem cell population to prevent the possibility of the tumour metastasizing.

**Figure 3. Cancer stem cell-targeted chemotherapies**

Most chemotherapies treat tumours by killing rapidly dividing cells with limited proliferative potential. If cancer stem cells are less sensitive to these therapies, they survive the treatment and re-establish the tumour after initial regression. If chemotherapies can successfully target cancer stem cells, tumours are unable to maintain themselves and grow. Eventually the tumours cannot generate new cells and will degenerate.

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1.2.5 Stemness genes

One of the important properties of stem cells is the strict regulation of proliferation, differentiation and self-renewal. The genes within pathways associated with these processes are known as “stemness” genes. These genes include the Notch, Shh and Wnt signalling pathways that are involved in self-renewal and cancer proliferation, as mentioned previously [20-22]. Studies also have shown that BMI-1, which is a member of the polycomb family of genes that regulate chromatin remodelling and act as transcriptional repressors, has been associated with normal stem-cell function and cancer. The gene has been recognized to be necessary for neural stem cell renewal [29].

An important gene for stemness is the hTERT gene, which encodes for the enzyme telomerase. The enzyme is a reverse transcriptase that maintains the length of telomeres, which are DNA-repeats at chromosomal ends that gradually shorten with cell divisions [30]. Since stem cells are long-lived, it is important for these cells to maintain and slow down telomere shortening to prevent chromosomal damage throughout their life-span. Therefore telomerase activity is often higher in stem cells compared to somatic cells, as evident in the haematopoietic stem cells [31]. Telomerase activity has also been associated with self-renewal potential in haematopoietic cells [32].

Another important stemness property is quiescence, or the low division rate of the stem cells. Stem cells proliferate when required by environmental factors, such as to repair tissue damage [11, 17]. Therefore, during most of their lifespan, stem cells will remain dormant and not divide. Genes that are active during proliferation will thus be lower in
expression when compared to other somatic cells. For example, MCM7, a member of the mini-chromosome maintenance family of proteins, is an essential component of the replication helicase complex required for DNA replication. Its expression is required during the cell cycle, but in quiescent cells (G0) it is found to be absent [33]. Therefore expression of MCM7 is a useful biomarker for proliferation, as shown in prostate cancer [34, 35].
1.3 Side population

1.3.1 Stem cells and side population

Currently, most methods used to define and isolate adult and cancer stem cells involve the use of cell surface markers and flow cytometry, such as in the studies by Al-Hajj and Singh [9, 10]. Some cell surface markers that have been used to identify stem cells and cancer stem cells are CD34^+ (haematopoietic) [8, 36], CD24^+CD44^+ (breast) [9], and CD133^+/nestin^+ (neural) [10]. However, the process of testing each individual potential marker would be inefficient. There has been a development in isolating stem cells that uses a more general method, which takes advantage of a physiological property of these cells. Studies have demonstrated that adult stem cells can be identified by their ability to actively efflux a fluorescent molecule, the Hoechst 33342 dye, resulting in a phenotype termed the “side population” (SP). The SP, first described by Margaret Goodell [37], is a small subpopulation of cells with enriched stem cell activity that shows a distinct “low” Hoechst 33342 dye staining pattern. Later studies attributed this phenotype to expression of ABCG2, an ATP-binding cassette (ABC) transporter [38]. Concurrent studies have demonstrated SP cells in human cancers of different origins, including acute myeloid leukemia [39, 40], neuroblastoma [41], and glioma [42]. SP cells have also been identified in head and neck squamous cell carcinoma [43], hepatocellular carcinoma [44], ovarian cancer [45], breast cancer and prostate cancer [46].

In these studies, researchers performed experiments to identify the SP by using and modifying Hoechst 33342 dye efflux assay to optimize for their particular cancer of
interest. They established that the SP disappeared by the use of an ABC transporter inhibitor, such as verapamil or reserpine in experiments on glioma cell line by Kondo [42], breast and prostate cell lines by Patrawala [46], and hepatocellular cell lines by Chiba [44]. Kondo study showed that SP cells can generate both the SP and non-SP cells, whereas non-SP cells generated mainly non-SP cells. Experiments by Patrawala showed preferential expression of stemness genes, including Notch-1, in SP cells compared to non-SP cells. The researchers in these studies transplanted the SP cells and the non-SP cells into immuno-compromised mice and saw an increase in tumourigenicity in SP. As little as $1 \times 10^3$ SP cells from hepatocellular cell line consistently formed tumours while up to $1 \times 10^6$ non-SP cells from the same cell line did not form tumours [44]. In prostate cancer cell lines, $1 \times 10^2$ SP cells were needed to form tumours in immuno-compromised mice while $3 \times 10^5$ non-SP cells were needed to form a tumour. In breast cancer cell lines, $1 \times 10^3$ SP cells formed tumours while $1 \times 10^4$ non-SP cells formed tumours. The increase in tumourigenicity in the SP cells of these studies proved that the SP contained an enriched population of tumour initiating cells. These results have suggested that the SP may be a source of cancer stem cells.

1.3.2 Hoechst 33342 Dye

Hoechst 33342 dye is a bis-benzimide molecule that fluoresces when excited by ultraviolet light [47] (Figure 4). The molecule was discovered to bind to the minor groove of double-stranded DNA. Since the dye bound proportionally to the amount of cellular DNA, studies were performed to isolate and characterise populations of cells at different stages in the cell cycle using flow cytometry along with Hoechst dye staining. It was only later discovered that stem cells exhibited lower levels of Hoechst 33342 dye
fluorescence when the relationship between cell cycle and stem cell potential was investigated in bone marrow [48]. This led to the isolation and characterisation of haematopoietic stem cells using the SP method by Goodell et al. [37].

The dye diffuses across the membrane at a limited rate through a carrier mediated process that has not been extensively studied [47]. When incubated at 37°C, concentration of dye in the medium decreases with time as the amount of dye absorbed by the cell increases. This continues until equilibrium is reached between the two diffusion rates, which usually occur at approximately 90 minutes. Unlike the remaining cells of the population, the SP cells extrude the dye against the concentration gradient and emit less fluorescence. Modifying the initial concentration of the dye and the cell concentration can affect the percentage of SP cells since this changes the amount of dye available to the sample. Coincubation of ABC transporter inhibitors, such as verapamil or reserpine, prevents the efflux of Hoechst 33342 dye. The SP cells are not able to pump out the dye and emit the same fluorescence as the remaining population, which results in the disappearance of the distinct SP tail [37, 47].

Ultraviolet light is used to excite the dye and emission is detected at both blue (402 to 446 nm) and red (650 to 670 nm). Since the Hoechst 33342 dye binds directly to DNA, the molecule disrupts DNA replication during cell division. Therefore, one of the major drawbacks to this dye efflux technique is the issue of cell viability. The side population studies involved using the Hoechst 33342 dye at low concentrations below levels that can affect cell function [47].
1.3.3 ATP-binding cassette (ABC) transporters

The ABC transporters represent a family of transmembrane proteins that actively use ATP to drive the transport of various molecules across cell membranes, including metabolic products, lipids, sterols and drugs [49]. ABC transporters are expressed in a variety of different organisms. While the structures of these proteins have been extensively studied, the exact method of function is not entirely known except for the role of ATP as the energy molecule. In humans, there are 49 known ABC transporters classified into seven subfamilies. The functions of these transporters range from transportation of cholesterol and other steroids to toxin secretion and ion transport. In cancer, there are three ABC transporter encoding genes in stem cells that represent the principle multidrug-resistance genes identified in tumour cells. These genes are ABCG2, ABCB1, and ABCC1, which encode the proteins BCRP1 (breast cancer resistance protein), MDR1 (multidrug-resistance), and MRP1 (multidrug-resistance-associated protein) respectively [50].

As mentioned in section 1.3.1, a study by Zhou et al. attributed the SP phenotype to expression of ATP-binding cassette (ABC) transporters, particularly the ABCG2 transporter (also known as BCRP1) [38]. In this study, they showed high levels of ABCG2 expression in primitive murine haematopoietic stem cells. They also
demonstrated that expression of ABCG2 directly conferred the SP phenotype by transfecting Saos-2, an osteosarcoma cell line that did not express the ABCG2. Transfected clones readily expelled Hoechst dye and efflux activity was inhibited by reserpine. As well, inactivating the plasmid with a mutation also abolished efflux activity. Using a monoclonal ABCG2 antibody, they showed strong correlation between ABCG2 expression and the SP phenotype. Finally, they determined that enforced expression of ABCG2 inhibited haematopoietic development in bone-marrow cells transduced with a retroviral vector, HaBCRP. Their result showed that BCRP1/ABCG2 gene was an important determinant in the SP phenotype.
1.4 Hypothesis and Objectives

The goal of my thesis was to determine the existence of side population cells in human lung cancer. SP was detected in human cancers of different epithelial origins and may likely exist in lung cancer. I hypothesized that the SP phenotype exists in lung cancer and the SP fraction represents an enriched population of tumour-initiating lung cancer stem cells.

The objectives of my research project were:

(i) To isolate the SP fraction from human lung cancer cell lines using the Hoechst 33342 efflux assay.

(ii) To characterise the SP fraction for stem cell-like properties.

(iii) To identify the SP in human lung tumours.

There were four specific aims that I achieved to complete my first two objectives. Firstly, I had to determine the existence of SP in established human lung cancer cell lines maintained in long-term culture by using modified published experimental protocols of the Hoechst 33342 dye exclusion assay. The cell lines chosen represented all three major types of non-small cell lung carcinomas: H23, H441, A549 (adenocarcinomas); HTB58, H2170 (squamous cell carcinomas); and H460 (large cell carcinoma). The second aim was to examine the expansive properties of the SP fraction by determining if SP cells were able to regenerate both SP and non-SP cells in culture, similar to experiments by Kondo [42]. Thirdly, molecular characterisation of stem cell-like properties was performed using quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR) to analyze mRNA expression of stem cell related genes. Lastly, I assessed the
functional properties of stem cell-like properties including invasiveness, chemotherapeutic resistance, and tumour-reinitiating capacity of SP cells. The in vivo experiments were performed to determine the differences in tumourigenicity between the two populations, similar to studies by Kondo [42], Patrawala [46], and Chiba [44]. Finally, I also examined for differences in expression of cell surface markers between SP and non-SP cells using known stem cell surface markers for other tissues. The cell surface markers examined were CD24 and CD44 (breast), CD34 (leukaemic), and nestin (neural). These markers were chosen due to their success in cancer stem cell studies, such as the potential marker signature CD24+/CD44+ for breast cancer stem cells [9].

The third objective was completed by using the same Hoechst 33342 dye efflux assay on 16 human clinical lung tumours. The clinical resections of lung tumours were digested and treated with a Percoll™ density gradient medium to separate epithelial cells from non-epithelial cells. Using the same Hoechst 33342 dye efflux protocol, the clinical samples were analyzed by FACS for SP. The results from this objective can be used for further work and raises important therapeutic implications.
2.0 MATERIALS AND METHODS

2.1 Cell culture and maintenance

Human tumour cell lines used were lung adenocarcinomas A549, NCI-H23 and NCI-H441, squamous lung carcinomas HTB-58 and NCI-H2170, and large cell lung carcinoma NCI-H460. All cell lines were obtained from American Type Culture Collection (ATCC, Manassas, Virginia). H23, H441, H2170 and H460 cells were cultured in RPMI medium 1640. A549 and HTB-58 cells were cultured in Ham’s F12 medium and Eagle’s minimum essential medium (MEM), respectively. All media contained 2 mM L-glutamine and were supplemented with 1% penicillin/streptomycin and 10% foetal bovine serum (FBS, Invitrogen Gibco-BRL, Burlington, Ontario). Cell lines were incubated in a humidified incubator at 37°C supplied with 5% carbon dioxide. Cells were routinely maintained in 75 cm² tissue culture flasks (BD Biosciences Discovery Labware, Oakville, Ontario) and were harvested by 0.25% trypsin-EDTA (Invitrogen-Gibco-BRL) treatment at roughly 80% confluent growth (logarithmic phase) for SP analysis. Cell counts were conducted using a cell haemocytometer counting chamber. Cells were re-suspended in appropriate media and 10 μL aliquot of the suspension was added to the haemocytometer. Cells within the four corner quadrants were enumerated and represented a cell count of $1 \times 10^4$ cells/mL.
2.2 Side population analysis

2.2.1 Hoechst dye efflux assay

The cell suspensions were labelled with Hoechst 33342 dye (Molecular Probes-Invitrogen, Carlsbad, California) using the methods described by Goodell et al with modifications [37]. Hoechst 33342 dye was dissolved at 1 mg/mL in ddH2O and filter sterilized using 0.2 µM filter. The stock solution was frozen in 50 µL small aliquots prior to usage at 1:200 for a final concentration of 5 µg/mL. Reserpine was prepared by dissolving the drug in DMSO at 30.435 mg/mL and used at 1:1000 for a final concentration of 50 µM. Cells were resuspended at 1 x 10^6 cells/mL in pre-warmed Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen Gibco-BRL) with 2% foetal calf serum (FCS, Invitrogen Gibco-BRL) and 10 mM HEPES buffer (Invitrogen Gibco-BRL). Hoechst 33342 dye was added at a final concentration of 5 µg/mL in the presence or absence of reserpine (50 µM) and the cells were incubated at 37°C for 90 minutes with intermittent shaking. At the end of the incubation, the cells were washed with ice-cold Hank’s Balanced Salt Solution (HBSS, Invitrogen Gibco-BRL) with 2% FCS and 10 mM HEPES, centrifuged down at 4°C, and resuspended in ice-cold HBSS containing 2% FCS, 10 mM HEPES and 2 µg/mL propidium iodide (PI, Molecular Probes-Invitrogen). The cells were filtered through a 40 µm-cell strainer (Becton Dickinson, Mississauga, Ontario) to obtain single cell suspension and kept on ice prior to sorting.

2.2.2 Fluorescence activated cell sorting (FACS)

Analyses and sorting were performed on a FACS Vantage SE (Becton Dickinson, Franklin Lakes, New Jersey). Propidium iodide is a membrane impermeant fluorescent
molecule that binds to DNA and generally excluded from viable cells. PI was measured using absorption maximum of 488 nm (535 nm when bound to DNA) and emission maximum of 590 nm (617 nm) to gate viable cells. The Hoechst 33342 dye was excited with ultraviolet (UV) light at 357 nm and its fluorescence was dual-wavelength analyzed at 402-446 nm, which represents blue, and 650-670 nm, which represents red. The cells were immediately analyzed after Hoechst dye incubation step and sorted into collection tubes with the regular culturing media according to the cell line (see section 2.1). The side population (SP) sorting gates were established by first defining a viable gate on the flow cytometer by collecting PI negative cells to exclude the dead cells (see Figure 5a). Approximately $5 \times 10^4$ events within the viable gate are then collected and analyzed for blue and red fluorescence of the Hoechst 33342 dye to clearly define the SP population with low Hoechst 33342 dye staining. A new gate was established to include this population as the SP and another gate was created to include the main population designated as the non-SP (Figure 5b). The SP was gated according to the disappearance of the tail in reserpine treated samples. The software FlowJo version 6.4.7 was used to analyze the data in the same approach as the FACS collection method.

Figure 5. (a) FACS profile showing gating of viable cells (b) FACS profile depicting distinct SP tail in population
2.3 Molecular characterisation of side population cells

2.3.1 RNA extraction and reverse transcription

Cells from the SP and non-SP population were sorted and total RNA was extracted using the RNEasy Micro kit (Qiagen, Mississauga, Ontario). Roughly $1 \times 10^5$ cells from each population was collected for lysis by guanidine-isothiocyanate from the extraction kit. Total RNA was extracted using the spin column-based silica-gel–membrane purification according to manufacturer's instructions. Total RNA was eluted into DEPC water (0.1% diethylpyrocarbonate in milli-Q water). The quantity and purity of total RNA were evaluated by the optical density at 260 nm ($A_{260}$) and 280 nm ($A_{280}$) using an UV spectrophotometer (Hitachi U-2000, Tokyo, Japan). The following formula is used to quantify the amount of total RNA: concentration in µg/mL = $A_{260}$ x weight per optical density x dilution factor. The purity of the total RNA is determined by the ratio between $A_{260}$ and $A_{280}$. To remove any traces of residual DNA, the total RNA was treated with 2 µL 10X DNase I buffer and 1 µL of DNase I to each aliquot of RNA (1 µg) (Invitrogen, Gibco-BRL) for 20 minutes at 37°C. The reaction was stopped by adding 2 µL DNase Inactivation Reagent. The isolated total RNA was subsequently reverse-transcribed to cDNA using random hexamers and Superscript II RT enzyme (Invitrogen) according to manufacturer's instructions. Each sample consisted of 10 µL 2X RT reaction mix, 2 µL RT enzyme mix, and 8 to 10 µL of mRNA (up to 1 µg). The PCR reactions were incubation at 25°C for 10 minutes followed by 50°C for 50 minutes. The reaction was terminated at 85°C for 5 minutes. One µL of *E. coli* RNase H was added to each sample and incubated at 37°C for 20 minutes. cDNA samples were stored at -20°C until used for real-time RT-PCR.
2.3.2 Primer design

DNA and mRNA sequences of genes were obtained from GenBank Release 158 at the NCBI website (http://www.ncbi.nlm.nih.gov/Genbank/) for primer design. Forward and reverse primers were designed using the primer analysis software Primer Express version 2.0 (Applied Biosystems, Foster City, California). Using this software, primers were chosen for optimal specificity and performance in real time RT-PCR. The melting temperature of the primers was between 56°C to 60°C with a length about 20 to 22 base pairs and a penalty index less than 100. Primers were designed to generate a PCR product of less than 200 base pairs. Table 1 shows a list of all primer pairs that generated single specific PCR products of the desired length for RT-PCR.

Table 1. List of primer pairs designed for real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>5' Primer</th>
<th>3' Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCG2</td>
<td>TGG GAC TGG TTA TAG GTG CCA</td>
<td>TGG TTG GTC GTC AGG AAG AAG</td>
</tr>
<tr>
<td>ABCA2</td>
<td>TCA AGA CAG GGC GTT CAG TG</td>
<td>GCG ACC GTT CAC CAT GAT G</td>
</tr>
<tr>
<td>MDR1</td>
<td>GGC CTA ATG CCG AAC ACA TT</td>
<td>AGG CTC AGT CCC TGA AGC AC</td>
</tr>
<tr>
<td>MRP1</td>
<td>CGG GCC GCA GAT CTT AAA G</td>
<td>GGC AGT GAC AAA CAG CAG CA</td>
</tr>
<tr>
<td>hTERT</td>
<td>GCC GAA GAC AGT GGT GAA CT</td>
<td>AGC TGG AGT AGT CTC TCT GC</td>
</tr>
<tr>
<td>MCM7</td>
<td>CCT CGC AGC CAG TAC ACA A</td>
<td>GCC CCA CCC TCT AAG GTC A</td>
</tr>
<tr>
<td>BMI-1</td>
<td>CCT CAT CCA CAG TTT CCT CAC A</td>
<td>TGA TTT TCG AGG TCT ATT GGC A</td>
</tr>
<tr>
<td>NOTCH1</td>
<td>TCC TGA TCC GGA ACC GAG</td>
<td>CGT CGT GCC ATC ATG CAT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>AAA TTC CAT GGC ACC GTC AA</td>
<td>CAG TCT TCT GGG TGG CAG TGA</td>
</tr>
<tr>
<td>MRP2</td>
<td>TGA GCA CCA GCA GCG ATT T</td>
<td>TTG CAA GCC ACC TGT TGG A</td>
</tr>
<tr>
<td>MRP3</td>
<td>GAG TCG CT TCA TGG TCT TGC</td>
<td>TGA TGC GCG AGT CCT TCA</td>
</tr>
<tr>
<td>MRP4</td>
<td>TCA TCG TT TCA TGG TCT TGC</td>
<td>AAG ATC CCC ATG AGC GTG C</td>
</tr>
<tr>
<td>MRP5</td>
<td>TTT GAA GCA CCT GCG AGA AT</td>
<td>TCG GTA CCT CAT CTC TGC GTT</td>
</tr>
<tr>
<td>MRP6</td>
<td>AAC ATC CCC ATG AGC GTG A</td>
<td>TTG GAA GCA CCT GCC AGA AT</td>
</tr>
<tr>
<td>MRP7</td>
<td>GCT GCA GGA AGA AAG CAA GAA</td>
<td>GCC TTG CAT GAG AAG CAG AGA</td>
</tr>
<tr>
<td>MRP8</td>
<td>CTT CTT CGT GGT GCT CAT CTA GT</td>
<td>TTG CTC TCT CGG CTA TTG</td>
</tr>
<tr>
<td>MRP9</td>
<td>TGG CCA AGC TCA ATT CAG C</td>
<td>TTT GAT CAG CCT GAT GCA GGT</td>
</tr>
</tbody>
</table>
2.3.3 Real-time RT-PCR

Real-time reverse transcriptase-PCR was performed using primers shown in Table 1. Reactions were performed with SYBR Green Real-Time Core Reagents (Applied Biosystems, Foster City, California) according to manufacturer's instructions on the ABI Prism 7900 Sequence Detection System (Applied Biosystems). Total reaction volume was adjusted proportionally to 15 μL per reaction. Each reaction contained 7.5 μL of 2X SYBR Green PCR buffer, 1.5 μL of 2 μM forward and reverse primers, 2.0 μL diluted cDNA (24 ng starting total RNA) from the RT reaction as described in section 2.3.1, and 2.5 μL of RNase-free DEPC PCR water. The critical threshold, or C_T, is established as the point at which fluorescence crosses an arbitrary value set at 10 standard deviations above mean fluorescence generated during baseline cycles. Cycling was performed using the default conditions of the ABI Prism 7900 SDS Software version 2.0 with a C_T baseline default set at 3 to 15. The thermal cycling conditions comprised of four stages. The first stage consisted of the initial amplification step that began the reaction by heating the PCR plate at 50°C for 2 minutes. This was followed by a denaturation step at 95°C for 5 minutes. The next stage consists of the annealing and extension step which comprised of 40 cycles of 15 seconds at 95°C, 30 seconds at 58°C, and 30 seconds at 72°C. The final stage terminates the reaction by an incubation step of 15 seconds at 95°C, 15 seconds at 60°C, and 15 seconds at 95°C. PCR reactions were performed in triplicates to ensure reproducibility of the technique.
2.3.4 Relative quantification in real-time RT-PCR

Levels of mRNA expression were calculated by relative quantification real-time RT-PCR. $C_T$, the critical threshold, was determined automatically by the ABI Prism 7900 SDS 2.0 software as the second derivative maximum of the amplification curve. The mRNA expression is compared between the SP and non-SP population of cells. The relative expression of each mRNA was normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by subtracting the difference of the $C_T$ levels between our gene of interest and GAPDH using the following formula: $2^{-\Delta C_T}$, where $\Delta C_T = C_T \text{(gene of interest)} - C_T \text{(housekeeping gene)}$. The expression levels were plotted in GraphPad Prism 4 (GraphPad Software, San Diego, California).
2.4 Functional characterisation of side population cells

2.4.1 Tumour cell implantation experiments

*In vivo* experiments were performed in accordance with the institutional guidelines for the use of laboratory animals. The mouse model used was an in-house bred non obese diabetic severe combined immunodeficient mouse (NOD-SCID). Mice were housed locally in sterile conditions in the Animal Research Centre (ARC) and kept in individually ventilated caging systems (IVC's) supplied with sterile filtered ddH₂O and HEPA filtered air (Micro-VENT mouse cages, Allentown Caging Equipment Company, Allentown, NJ). Mice were housed with laboratory animal bedding (Bed-O-Cob, The Andersons, Maumee, OH) and standard laboratory rodent diet feed (PicoLab Rodent Diet 20, Canadian Lab Diets, Inc, Leduc, Alberta). SP and non-SP cells from H460, A549, and H441 were subcutaneously injected into four week old female mice using a limiting dilution assay. Groups of mice were inoculated with SP cells at $1 \times 10^5$, $5 \times 10^4$, $5 \times 10^3$ and $1 \times 10^3$ cells or non-SP cells at $1 \times 10^5$, $5 \times 10^4$ and $5 \times 10^3$ cells (three to four mice per group). Tumour growth was monitored every two days after 1st week of inoculation. The mice were sacrificed at day 60 or when the tumours grew to a maximum of 1000 mm$^3$. Tumour volume was calculated by the formula $0.52 \times \text{length} \times \text{width}^2$. Fold difference in tumourigenicity was calculated by the formula: (minimum number of non-SP cells needed to generate a tumour) / (minimum number of SP cells needed to generate a tumour).
2.4.2 Re-analysis of SP-derived tumours

The tumours were surgically removed, mechanically minced with surgical scissors and dissecting blade. The tissue was then incubated in DMEM + 10% FBS with 0.1 Wünsch units/ml collagenase (Roche) for a 3 hour digestion period. The collagenase was removed by centrifuge and digested tumours were sieved through a 100 μm-cell strainer (BD-Biosciences) to obtain single cell suspension. Cells were re-analyzed by the Hoechst 33342 dye efflux assay as described previously (see section 2.2.1 and 2.2.2).

2.4.3 Invasion assay

The cellular potential for invasiveness of SP and non-SP cells was determined using a 6-well Matrigel™ Invasion Chambers (BD Biosciences Discovery Labware). Cells were seeded into separate top chamber inserts at $2 \times 10^5$ cells per insert in serum-free DMEM and the bottom outer wells were filled with DMEM containing 5% FBS as chemo-attractant as shown in Figure 6. Cells were incubated at 37°C with 5% carbon dioxide for 48 hours, and then non-invading cells were removed by swabbing top layer of Matrigel™ with a Q-tip. The membrane containing invading cells were fixed with alcohol for 1 min then stained with haematoxylin for 5 min and mounted on glass slides. The entire membrane with invading cells was counted using a light microscope with a 40x objective. Increase of invasiveness was calculated by number of cells on membrane with SP cells divided by the number of cells on membrane with non-SP cells. Experiments were run in triplicates on three independent tests to ensure reproducibility.
2.4.4 Drug sensitivity assay

Chemotherapeutic drugs used in this experiment were obtained through the British Columbia Cancer Agency Pharmacy. The drug concentrations used were at IC$_{50}$ when 50% of cells are viable after treatment. These values were determined by adding the different concentration drugs to each unsorted cell line and plotting a dose-response curve. For each cell line, 5x10$^3$ cells were seeded in 96-well plate along with 100 μL of media and incubated for 24 hours at 37°C to allow cells to attach. A concentration range for the drug was then added to the wells in triplicates. For example, concentrations of etoposide added to A549 cells ranged from 1μM, 2μM, 5μM, 10μM, to 50μM while one triplicate set of wells were left untreated. The cells were then incubated for 48 hours at 37°C to allow the drug to act. Sensitivity was determined using a colourimetric MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) cell proliferation assay (CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS), Promega, Madison, WI, USA) according to manufacturer’s instruction. Twenty μL of substrate solution was added to cells and incubated for 2 hours at 37°C. The colour of the media changed from yellow to dark purple as viable cells broke down and converted the tetrazolium substrate to its formazan product. Absorbance
was measured at 490 nm for each well using a micro-plate reader (Dynex Technology, Chantilly, Virginia). The difference in intensity of the color change is determined by the amount of viable cells remaining within the wells. Percent viability calculated using the formula: \( \text{percent viability} = \frac{\text{absorbance of treated cells}}{\text{absorbance of untreated cells}} \times 100\% \). Cell viability decreased as drug concentration increased in a non-linear fashion. Examples of dose-response curve plot of several drugs on different cell lines are shown in Figure 7. Drug concentrations at which 50% cells survive are approximated and shown in Table 2 for the seven drugs on the six cell lines. FACS-sorted cells were counted by the trypan blue assay for viability and seeded in 96-well plate format with appropriate growth medium at 100 µl per well. After 24 hours of recovery, drugs were added at the \( IC_{50} \) concentration for each sorted cell line and incubated for another 24 hours. Drug resistance was represented as percent viability calculated using the formula: \( \text{percent viability} = \frac{\text{absorbance of treated cells}}{\text{absorbance of untreated cells}} \times 100\% \).
Figure 7. Examples of dose response curve of chemotherapeutic drugs on lung cancer lines.

(a) Etoposide on A549  
(b) Cisplatin on HTB58  
(c) Docetaxel on H23  
(d) Etoposide on H23

Table 2. IC\textsubscript{50} drug concentration values for lung cancer cell lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Cisplatin</th>
<th>Etoposide</th>
<th>Gemcitabine</th>
<th>Vinorelbine</th>
<th>Docetaxel</th>
<th>Doxorubicin</th>
<th>Daunorubicin</th>
</tr>
</thead>
<tbody>
<tr>
<td>H460</td>
<td>10uM</td>
<td>20uM</td>
<td>200nM</td>
<td>400nM</td>
<td>400nM</td>
<td>500nM</td>
<td>500nM</td>
</tr>
<tr>
<td>HTB58</td>
<td>15uM</td>
<td>10uM</td>
<td>100nM</td>
<td>3uM</td>
<td>2uM</td>
<td>500nM</td>
<td>500nM</td>
</tr>
<tr>
<td>H2170</td>
<td>15uM</td>
<td>30uM</td>
<td>5uM</td>
<td>5uM</td>
<td>2uM</td>
<td>2uM</td>
<td>2uM</td>
</tr>
<tr>
<td>H441</td>
<td>50uM</td>
<td>20uM</td>
<td>500nM</td>
<td>5uM</td>
<td>1uM</td>
<td>2uM</td>
<td>2uM</td>
</tr>
<tr>
<td>H23</td>
<td>25uM</td>
<td>5uM</td>
<td>500nM</td>
<td>1uM</td>
<td>500nM</td>
<td>500nM</td>
<td>500nM</td>
</tr>
<tr>
<td>A549</td>
<td>10uM</td>
<td>50uM</td>
<td>5uM</td>
<td>500nM</td>
<td>500nM</td>
<td>500nM</td>
<td>500nM</td>
</tr>
</tbody>
</table>
2.5 Multiple cell-surface marker staining

The antibodies for the cell-surface markers CD24, CD34, CD44, and nestin were purchased as conjugates to either allophycocyanin (APC) or phycoerythrin (PE). Cells were first stained with the Hoechst 33342 dye for 90 min as described in section 2.2.1. Hoechst-stained cells were centrifuged and resuspended in HFN (Hank’s Balanced Salt Solution + 2% FCS + 0.05% NaN₃) + 5% human serum at a concentration of 5-7 x 10⁴ cells per µL. Sorted cells were co-stained with CD34-APC (BD-Biosciences) and Nestin-PE (Cedarlane labs, Hornby, Ontario, Canada) antibodies or with CD24-PE (BD-Biosciences) and CD44-APC (BD-Biosciences) antibodies at 10 µL per 1 x 10⁶ cells.

After incubation in the dark for 30 min on ice, 1 ml HFN was added to each tube and centrifuged. Cells were resuspended in 300 µL HFN with 2 µg/mL PI and analyzed using FACS. Staining profile for each marker was constructed within the SP and non-SP gates separately and compared.

2.6 Clinical lung tumour resection

Fresh tumour samples obtained within 60 minutes of surgery were kept on ice prior to being processed. Samples were rinsed with HBSS and mechanically minced into fragments less than 1 mm³ using surgical scissors and dissecting blade. The tissue was enzymatically digested for 4 hours at 37°C in a shaking incubator with 0.1 Wünsch units/mL collagenase (Roche, Mississauga, Ontario, Canada) in DMEM. After the enzymatic digestion period, collagenase was removed by centrifugation and the digested pellet was resuspended in fresh DMEM. The sample was further disaggregated through an 18½G needle with a 10 mL syringe and sieved through a 100 µm-cell strainer (BD-Biosciences) to obtain single cell suspension. To remove non-epithelial cells, the single
cell suspension was layered onto a 70% and 40% Percoll™ gradient (Amersham Biosciences, Piscataway, NJ) and centrifuged for 20 minutes at 2,500 rpm with no brake. Epithelial cells remaining at the 70% / 40% interface were then collected for SP analysis using the same protocol (see section 2.2.1 and 2.2.2). Cell viability was determined by trypan blue exclusion. Due to the low viable cell recovery after the digestion, the reserpine treatment could not be performed. The SP gate for clinical tumour samples were drawn according to the shape of the SP tail based on experiments in the cancer cell line samples.

2.7 Statistical analyses
Data were presented as the mean ± standard deviation (SD). To assess statistical significance of differences, an unpaired 2-tailed t-test (GraphPad Software Inc., San Diego, CA) was performed. P values <0.05 were considered significant, as indicated by asterisks.
3.0 RESULTS

3.1 SP phenotype is observed in human cancer cell lines
My project examined the existence of SPs in six human lung cancer cell lines by staining them with Hoechst 33342 dye to generate a Hoechst blue-red profile. As a control, reserpine was added to block the activity of Hoechst 33342 transporter, and the SP gate was defined as the diminished region in the presence of reserpine. The profile of H460 was shown as an example with SP and non-SP gates labelled (Figure 8a). Profiles of H23, H441, H2170, HTB58, A549 are shown with and without the presence of reserpine (Figure 8b to f, respectively). All six lung cancer cell lines contained a distinct fraction of SP cells in multiple sorts, ranging from averages of 1.5% (H23) to 6.1% (H441) of gated cells, as shown on Figure 9. SP percentages decreased significantly in the presence of reserpine, also shown on Figure 9.

3.2 SP regenerates both SP and non-SP
To compare the repopulation ability of lung cancer SP cells with non-SP cells, we cultured the sorted SP and non-SP cells separately under the same culture condition for two weeks before they were re-stained with Hoechst 33342 dye and reanalyzed using FACS. Both fractions were viable in culture, but the SP cells generated both SP and a non-SP with a fraction size comparable to the original population, whereas the non-SP cells produced mainly non-SP cells, with a significant decrease in the fraction of SP cells. The profile of SP and non-SP H460 cells reanalyzed is shown in Figure 10a and b. Regeneration of SP and non-SP cells from SP in six cell lines is shown in Figure 10c.
Figure 8. SP in human lung cancer cell lines

(a) H460, (b) H23, (c) H441, (d) H2170, (e) HTB58 (f) A549 were stained with Hoechst 33342 dye in the presence (Right) or absence (Left) of 50μM reserpine and analyzed by flow cytometry. The SP, which disappears in the presence of reserpine, was gated and shown as a percentage of the whole viable cell population.
Figure 8 continued.

d) H2170 without reserpine
2.5%

H2170 with reserpine


e) HTB58 without reserpine
4.2%

HTB58 with reserpine


f) A549 without reserpine
4.2%

A549 with reserpine
Figure 9. Percent of the SP gated with or without addition of reserpine in all six lung cancer cell lines repeated in experiments (triplicate or more). Asterisks indicate significant difference (p value < 0.05 in an unpaired 2-tailed t-test).
Figure 10. SP regenerates both SP and non-SP

(a) SP and non-SP cells sorted from H460 were cultured for two weeks before re-staining with Hoechst dye and reanalyzed. The SP (left) generated both a significant SP and non-SP fraction comparable to the original sort, while the non-SP (right) was mostly non-SP.

(b) Percentages of SP regenerated from the SP and non-SP fraction for other lung cancer cell lines. Asterisks indicate significant difference (p value < 0.05 in an unpaired 2-tailed t-test)
3.3 Molecular characterisation of side population cells

3.3.1 ABC transporters were up-regulated in SP

Expression of ABC transporters has been shown in primitive cells and associated with its capacity to export a broad range of cytotoxic drugs (reviewed [44]). In particular, ABCG2 has been implicated in the high Hoechst 33342 dye efflux capacity that marks the SP phenotype. Using a real-time RT-PCR assay, we determined the relative mRNA expression level of human ABC transporters in the SP and non-SP of lung cancer cell lines. Twelve ABC transporters were studied, including the four major drug transporters (ABCA2, MDR1, MRP1, ABCG2) and related subfamily members (MRP2 to 9). Consistent with previous reports, ABCG2 was elevated in the SP of all cell lines (Figure 11a). In addition, the SP fraction expressed other drug transporters at a significantly higher level than the non-SP in H460 (ABCA2, MDR1, MRP1), A549 (ABCA2, MRP1), HTB58 (ABCA2, MDR1, MRP1), H441 (MDR1), and H2170 (MDR1, MRP1) (Figure 11b, c, and d). Several related subfamily members were also found in higher levels in the SP compared to the non-SP. Relative expression levels of MRPs 2 to 9 in SP and non-SP cells of all six lung cancer cells are plotted on Figure 12a to f.
Figure 11. mRNA expression levels of the ABC transporters in SP and non-SP.
Relative expression levels of (a) ABCG2, (b) ABCA2, (c) MDR1, (d) MRP1 in each cell line were determined by real-time RT-PCR. Expression levels were normalized to the housekeeping gene GAPDH. Asterisk indicates statistical significance (p < 0.05 in an unpaired 2-tailed t-test).
Figure 12. mRNA expression levels of the other MRP-subfamily ABC transporters in SP and non-SP.

Relative expression levels of MRPs 2 to 9 in SP and non-SP of H460 (a), H23 (b), HTB58 (c), A549 (d), H441 (e), H2170 (f) determined by real-time RT-PCR. Expression levels were normalized to the housekeeping gene GAPDH.
3.3.2 hTERT levels were elevated in SP

To elucidate possible differences in telomerase expression between SP and non-SP, mRNA levels of hTERT (human telomerase reverse transcriptase), the catalytic subunit of telomerase and limiting factor for telomerase activity, were measured and compared. As shown in Figure 13a, hTERT expression was higher in SP cells for all six cell lines tested.

3.3.3 MCM7 expression was lower in the SP

MCM proteins (mini-chromosome maintenance) are essential components of the replication helicase complex. They have been shown to be useful markers that reflect the cell cycle state. The SP showed lower mRNA expression levels of MCM7, a member of the MCM family and a proliferation marker, in all six cell lines tested (Figure 13b).

3.3.4 BMI-1 expression was higher in the SP

BMI-1 is an important gene that regulates normal stem cell function and necessary for stem cell renewal. mRNA of BMI-1 was expressed higher in four of six cell lines (H460, H23, HTB58 and H441) in the SP compared to the non-SP, as shown in Figure 13c.

3.3.5 Notch-1 expression level was elevated in SP

Expression of Notch-1, a gene involved in stem cell self-renewal and inhibition of differentiation, was compared between SP and non-SP cells. Figure 13d shows five of six cell lines (H460, A549, H23, HTB58, and H441) with increased mRNA expression in SP cell compared to non-SP cells.
Figure 13. mRNA expression levels of the stemness genes in SP and non-SP.

Relative expression levels of (a) hTERT, (b) MCM7, (c) BMI1, (d) NOTCH1 in each cell line were determined by real-time RT-PCR. Expression levels were normalized to the housekeeping gene GAPDH. Asterisk indicates statistical significance (p < 0.05 in an unpaired 2-tailed t-test).
3.4 Functional characterisation of side population cells

3.4.1 SP were more tumourigenic in vivo

To test whether SP cells were enriched for tumourigenic cells, various numbers of SP and non-SP cells from H460, A549, and H441, cell lines known to give rise to tumours in vivo, were subcutaneously injected into mice and monitored for tumour development. As shown in Table 3 and Figure 14a, H460 non-SP cells give rise to new tumours at $1 \times 10^5$ in only one out of four mice tested. However, SP cells could form a tumour when only $5 \times 10^4$ cells (3/3 animals) were inoculated suggesting that H460 SP were enriched for tumour-initiating cells by at least 2-fold. This enrichment-fold is likely an underestimation because at the same injection dose ($1 \times 10^5$ cells), the tumour generated by the SP (1350 mm$^3$) is 15-fold larger in volume than that of the non-SP (88 mm$^3$). For A549, SP cells gave rise to tumours with as little as $1 \times 10^3$ cells (2/4 animals), while at least $5 \times 10^4$ non-SP cells were needed to form a tumour (3/4 animals). Hence A549 SP was significantly enriched for tumourigenic cells by approximately 50-fold. H441 SP cells generated a tumour with $5 \times 10^3$ cells, compared to at least $5 \times 10^4$ needed for non-SP to form a tumour, demonstrating that H441 SP was at least 10-fold enriched in tumourigenicity. Reanalysis of SP-derived tumours by flow cytometry showed that, similar to SP cultured in vitro, SP cells under in vivo conditions also have the capacity to regenerate the SP and non-SP fractions (Figure 14b).
Figure 14. SP cells were more tumourigenic.
(a) Representative subcutaneous tumours due to injection of H460, A549, and H441 SP cells compared to non-SP injection.
(b) Re-analysis of SP-derived tumour by the Hoechst dye efflux assay.

Figure 15
b)
Table 3. Tumourigenicity of SP cells in NOD/SCID.

Table shows number of tumours formed in groups of mice when injected with cells from H460, A549 and H441 cell lines in limiting dilution method, $1 \times 10^3$, $5 \times 10^3$, $5 \times 10^4$, and $1 \times 10^5$. Brackets indicate mean tumour volume in mm$^3$.

<table>
<thead>
<tr>
<th>Cell numbers for injection (tumour volume mm$^3$)*</th>
<th>$1 \times 10^3$</th>
<th>$5 \times 10^3$</th>
<th>$5 \times 10^4$</th>
<th>$1 \times 10^5$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>H460</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SP cells</td>
<td>0/4</td>
<td>0/4</td>
<td>3/3 (1270)</td>
<td>3/3 (1350)</td>
</tr>
<tr>
<td>Non-SP cells</td>
<td>(-)</td>
<td>0/4</td>
<td>0/4</td>
<td>1/4 (88)</td>
</tr>
<tr>
<td><strong>A549</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-SP cells</td>
<td>(-)</td>
<td>0/4</td>
<td>3/4 (18)</td>
<td>4/4 (126)</td>
</tr>
<tr>
<td><strong>H441</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SP cells</td>
<td>0/4</td>
<td>2/4 (379)</td>
<td>4/4 (421)</td>
<td>3/3 (973)</td>
</tr>
<tr>
<td>Non-SP cells</td>
<td>(-)</td>
<td>0/4</td>
<td>4/4 (204)</td>
<td>4/4 (676)</td>
</tr>
</tbody>
</table>

(-) dose not tested

* Formula used for tumour measurements: $0.52 \times$ length x (width)$^2$
3.4.2 SP displayed increased invasiveness

To investigate possible differences in invasiveness between SP and non-SP, an *in vitro* Matrigel invasion assay was performed on sorted cells of each cell line. Figure 16 shows that SP cells were more invasive in H460 (2.3x), A549 (2.3x), HTB-58 (1.6x), H441 (1.7x) and H2170 (2.0x). Both SP and non-SP cells from H23 were non-invasive.

![Matrigel Invasion Assay](image)

**Figure 16.** Invasiveness as measured by the Matrigel assay. $4 \times 10^5$ SP or Non-SP cells were seeded and incubated for 72 hours. Bars represent the number of cells invaded across the membrane. Asterisk indicates statistical significance ($p < 0.05$ in an unpaired 2-tailed t-test).
3.4.3 SP showed higher resistance to chemotherapeutic drugs

Since drug resistance is a common characteristic of normal and cancer stem cells, my project tested whether the SP show heightened resistance to drugs commonly used in chemotherapy. Sensitivity assays using seven chemotherapeutic drugs were performed on SP and non-SP cells of H460, HTB58, H2170 and H441. A549 cells showed no difference between SP and non-SP population, while both H23 SP and non-SP cells did not recover from drug treatment. Table 4 shows the four of the cell lines that exhibited higher resistance to chemotherapeutic drugs in the SP compared to non-SP. Values represent percent viability calculated using the formula: (absorbance of treated cells) / (absorbance of untreated cells) x 100%. In particular, SP was resistant to all seven drugs tested for H460, to cisplatin and etoposide for HTB58, to etoposide, gemcitabine, doxorubicin and daunorubicin for H2170, and to etoposide, vinorelbine and docetaxel for H441.
Table 4. Side population shows high resistance to chemotherapeutic drugs

<table>
<thead>
<tr>
<th></th>
<th>Cisplatin</th>
<th>Etoposide</th>
<th>Gemcitabine</th>
<th>Vinorelbine</th>
<th>Docetaxel</th>
<th>Doxorubicin</th>
<th>Daunorubicin</th>
</tr>
</thead>
<tbody>
<tr>
<td>H460 SP</td>
<td>42.0±4.9</td>
<td>67.3±3.8</td>
<td>33.95±7.4</td>
<td>31.7±2.1</td>
<td>49.4±3.7</td>
<td>53.5±6.9</td>
<td>32.3±2.7</td>
</tr>
<tr>
<td>H460 non-SP</td>
<td>30.9±7.2</td>
<td>59.2±3.5</td>
<td>26.9±4.2</td>
<td>23.8±2.7</td>
<td>37.2±5.0</td>
<td>29.5±3.1</td>
<td>17.8±1.6</td>
</tr>
<tr>
<td></td>
<td>p=0.007*</td>
<td>p=0.002*</td>
<td>p=0.05*</td>
<td>p=0.0001*</td>
<td>p=0.002*</td>
<td>p=0.005*</td>
<td>p=0.001*</td>
</tr>
<tr>
<td>HTB58 SP</td>
<td>64.6±8.8</td>
<td>57.3±5.2</td>
<td>52.4±4.8</td>
<td>41.6±2.9</td>
<td>58.2±6.7</td>
<td>48.5±2.3</td>
<td>46.6±3.5</td>
</tr>
<tr>
<td>HTB58 non-SP</td>
<td>52.1±5.0</td>
<td>49.3±4.4</td>
<td>53.6±6.7</td>
<td>45.8±4.4</td>
<td>64.7±7.1</td>
<td>55.2±6.7</td>
<td>48.4±4.1</td>
</tr>
<tr>
<td></td>
<td>p=0.04*</td>
<td>p=0.02*</td>
<td>p=0.7</td>
<td>p=0.09</td>
<td>p=0.1</td>
<td>p=0.2</td>
<td>p=0.6</td>
</tr>
<tr>
<td>H2170 SP</td>
<td>33.3±3.1</td>
<td>92.6±5.8</td>
<td>50.4±3.7</td>
<td>75.4±8.6</td>
<td>60.7±3.6</td>
<td>80.0±8.8</td>
<td>53.4±3.9</td>
</tr>
<tr>
<td>H2170 non-SP</td>
<td>20.2±0.7</td>
<td>42.0±3.7</td>
<td>23.9±0.6</td>
<td>71.6±5.5</td>
<td>63.4±4.9</td>
<td>34.9±1.6</td>
<td>28.7±3.0</td>
</tr>
<tr>
<td></td>
<td>p=0.0001*</td>
<td>p=0.009*</td>
<td>p=0.008*</td>
<td>p=0.2</td>
<td>p=0.5</td>
<td>p=0.01*</td>
<td>p=0.01*</td>
</tr>
<tr>
<td>H441 SP</td>
<td>52.3±3.9</td>
<td>88.5±9.7</td>
<td>72.5±8.1</td>
<td>80.9±4.9</td>
<td>80.2±5.7</td>
<td>60.3±1.0</td>
<td>57.7±5.1</td>
</tr>
<tr>
<td>H441 non-SP</td>
<td>53.9±2.4</td>
<td>78.0±6.9</td>
<td>71.1±6.9</td>
<td>68.9±7.0</td>
<td>74.2±5.7</td>
<td>60.4±5.7</td>
<td>60.5±3.5</td>
</tr>
<tr>
<td></td>
<td>p=0.3</td>
<td>p=0.01*</td>
<td>p=0.7</td>
<td>p=0.003*</td>
<td>p=0.03*</td>
<td>p=1.0</td>
<td>p=0.6</td>
</tr>
</tbody>
</table>

Values represent percent viability calculated using the formula: (absorbance of treated cells) / (absorbance of untreated cells) x 100%
Values are expressed as mean ± standard deviation
Experiments performed in triplicates in three independent tests (9 experiments in total)
Resistance quantified as percentage viability after drug exposure for 24 hours.
Statistical significance (p < 0.05 in an unpaired 2-tailed t-test) indicated by asterisks.
3.5 Staining of SP cells for other putative stem cell markers

To elucidate on the possible association of other putative stem cell markers to the lung cancer SP phenotype, cell lines stained by the Hoechst 33342 dye were additionally stained for CD24, CD34, CD44 and Nestin. However, there were no significant differences between the SP and non-SP for each individual marker (Table 5).

Table 5. Stem cell marker staining in SP and non-SP

<table>
<thead>
<tr>
<th></th>
<th>CD24</th>
<th>CD34</th>
<th>CD44</th>
<th>Nestin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SP</td>
<td>non-SP</td>
<td>SP</td>
<td>non-SP</td>
</tr>
<tr>
<td>H460</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A549</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H23</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HTB58</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H441</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H2170</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

0 - 25% stain (-), 26 - 50% (+), 51 - 75% (++), 76 - 100% (+++)
3.6 SP was present in clinical lung cancer samples

To see if clinical samples also contain an SP, 16 surgical resections from lung cancer patients were stained with Hoechst 33342 dye for FACS-analysis. As shown in Figure 17a and b, all samples tested showed a small SP (0.03% to 1.12%), demonstrating the presence of this population in clinical lung cancer similar to lung cancer cell lines.

Figure 17. SP was present in clinical lung cancer samples.
(a) Surgical resection of lung cancer patient #11 was digested by liberase collagenase overnight and subsequently stained by Hoechst 33342 for FACS-analysis.
(b) Percentage of SP found in clinical lung cancer samples.
4.0 DISCUSSION

4.1 Side population and cancer stem cells

This research supports the existence of an SP, a fraction enriched for stem cells in long-term cancer cell cultures. The data showed that the SP could be reliably detected under the experimental conditions used. For all six lung cancer cell lines, the SP was a distinct population that appeared in the FACS profile. The disappearance of the SP tail when incubated with reserpine confirmed the role of ABC transporters in the Hoechst 33342 dye efflux assay and was consistent with previous studies that used ABC transporter inhibitors to define the SP [39-46]. The ability to reliably isolate an SP in lung cancer cell lines allowed for subsequent studies on this distinct population for stem cell-like properties. The SP protocol was modified and tested repeatedly before it was optimized for lung cancer. Similar to previous studies, the incubation conditions that influenced the characteristics of the SP tail, such as length of time, temperature, cell concentration, and dye concentration were strictly controlled to achieve consistency in the results. The cells were also maintained at ice-cold temperatures to prevent additional dye efflux after staining. The temperature of the incubation greatly affected the staining of the cells. With a molecular weight larger than glucose, the dye molecule is believed to penetrate the cell through facilitated diffusion. Further issues with this assay included the concern for cell viability after incubation with Hoechst dye. Though the dye concentration levels used for the assay were well below cell toxicity levels, it may be relevant to determine whether cell viability affected the results. Estimation of cell viability by PI staining in FACS after dye incubation consistently yielded viability percentages of 80 to 90% for all six cell lines. When sorted SP and non-SP cells were plated into culture media, both
populations attached and proliferated at equal rates. It was further confirmed in chemotherapeutic experiments when SP and non-SP cells showed equivalent viability the mock (untreated) wells. It is clear that the dye toxic effects of the dye were minimal and did not affect the results of the experiments.

It is interesting to note the differences in the SP tail among the different cell lines. The SP tail was defined as the disappearance of the cells on the FACS profile for the reserpine-treated experiments. Studies on the SP tail have shown differences in stem cell properties for separate sections of the tail [51]. The cells that were less stained appeared on the lower tip of the SP tail and had higher dye efflux compared to the cells of the upper part of the SP tail. It was shown these cells in the lowest portion of the SP tail had the highest haematopoietic stem cell ability, as fewer cells were needed to reconstitute the bone marrow in a competitive transplantation experiment. This result clearly indicated the direct correlation between Hoechst 33342 dye efflux and stem cell potential, and the heterogeneous composition of the SP fraction further supported the hierarchal model of cancer stem cells. Some SP cells were more stem-like than other SP cells. The gating of the SP in lung cancer included the entire heterogeneous population, thus collecting SP cells with differing degrees of stemness.

The experiments from the re-analysis of the SP and non-SP yielded interesting results. The SP regenerated SP and non-SP similar to the original population as expected. However, the non-SP regenerated both SP and non-SP as well, which suggested that the non-SP could also contain some cancer stem-like cells even though SP percentage of the
non-SP was significantly lower than that of the original population. It is possible cancer stem-like cells may exist outside the SP tail and not possess the SP phenotype of higher dye efflux ability. Also, it has been suggested that origin of cancer stem cells may be from differentiated cancer cells. The existence of SP cells regenerating from the non-SP may allude to the possibility that non-SP cells can dedifferentiate and become more stem-like. However, further examination will be needed to prove this process.

### 4.2 In-vivo studies and tumourigenicity of SP cells

When injected into NOD-SCID mice, SP cells were found to be more tumourigenic than non-SP, thus indicating a significant enrichment of tumour-initiating cells in this small subpopulation. Even though the non-SP forms the majority of cells, the SP cells formed larger tumours and required fewer cells. This is consistent with the SP in other in-vivo tumourigenicity studies experiments performed glioma, hepatocellular carcinoma, and breast and prostate cancer cell lines [42, 44, 46]. The increase in tumourigenicity of SP cells is potentially important since effective curative therapy most likely depends on at least the successful eradication of these cancer-initiating SP cells. If these cells are less sensitive to conventional therapies, they may remain viable after treatment and re-establish the tumour even after initial regression. As observed by other investigators [41, 42], my project also found both in vitro and in vivo evidence for the SP to regenerate a population of cells comprised of both SP and non-SP, resembling the original unsorted population, thus demonstrating repopulating capacities similar to stem cells.

Interesting, the lowest number of SP cells required to form tumours varied from 1,000 cells to 50,000 cells depending on the cell line. This variation may be due to intrinsic
differences in tumourigenic potential between the different cell lines. One of the studies revealed as little as 200 SP cells from a prostate cancer cell line were needed to form a tumour [46]. The researchers inoculated prostate cancer SP cells within Matrigel™ to provide an environment with extra-cellular matrix proteins such as laminin, collagen, and proteoglycans, thus enabled more successful growth of tumours. If this procedure was adopted for my study on lung cancer, the tumourigenic studies may have yielded results where lung cancer SP cells form tumours with fewer cells than 1,000 cells. While these numbers are still far off from establishing that one putative cancer stem cell can form tumours identical to the original tumour, we must remember that the SP is only a technique for enrichment of stem-like cancer cells. The SP does not contain a pure population of cancer stem cells and some cancer stem cells may exist outside of the SP. Therefore, if cancer stem cells are inoculated as part of the non-SP fraction, they may increase the potential for tumours to form from that population.

The finding that lung cancer SP cells were more invasive than non-SP suggests that stemness may be related to invasiveness. Normal stem cells are known to be more mobile since they migrate to areas of tissue damage. Likely, there exists a population of stem-like cells within a lung tumour that is involved in the initiation of invasion. The concept of migrating cancer stem cells has many important implications. If cancer stem cells are responsible for invading and migrating to distant tissue sites, then the importance of targeting and neutralizing these cells is even greater. The final stage in malignancy is the metastasis and is the main cause of death for cancer patients. It is known that one of the crucial events to malignancy that occurs before metastasis is the
gain of migratory phenotype at the expense of epithelial-cell properties. This is referred as the epithelial to mesenchymal transition (EMT) [52]. This concept was proposed as a two-phase process in which “stationary” epithelial cancer stem cells acquire mesenchymal characteristics and become migrating cancer stem cells [53]. The combination of migratory and stemness properties integrates both tumour metastasis and initiation concepts into one cell, and potentially provides an explanation to tumourigenic progress. Since the putative cancer stem cell is more tumourigenic than other cancer cells, their added increase in mobility enables them to initiate tumours at distant sites from the primary tumour, and adds to the importance of developing cancer therapies that target these cells.

Furthermore, invasiveness may also be associated with the interaction between cancer stem cells and their niche. The niche consists of the supporting cells surrounding the cancer stem cells. A study by Kaplan et al. investigated the relationship of niche formation and metastasis [54]. They found that the initiation of a niche for metastasis was associated with haematopoietic progenitor cells derived from bone marrow. Secreted factors from different types of tumour cells into conditioned media directed these haematopoietic progenitor cells towards future preferred sites of metastasis to form cellular clusters. This remodeled the microenvironment of the preferred site into a more favourable, pre-metastatic niche before tumour cells were injected.

This concept that cancer stem cells drive both initiation of tumourigenesis and metastasis can explain the formation of tumour at distinct sites. A review by Li et al. proposed a
cancer stem cell model to describe metastasis and cancer stem cells [55] (Figure 18). Mutations in normal adult stem cells give rise to cancer stem cells. The self-renewal ability of these cancer stem cells along with further accumulations of oncogenic mutations causes the development of the primary tumour. The tumour is fuelled and sustained by the initial pool of cancer stem cells, and eventually secrete factors that form the pre-metastatic niche at distinct sites. The metastatic cancer stem cells are guided towards the niche at these distinct sites through chemo-attractants and other homing factors. When the cancer stem cells arrive, they are able to proliferate into a metastatic lesion, or enter a quiescent period until reactivated to promote expansion into a secondary tumour.

**Figure 18. Model for metastasis and cancer stem cells**

Stem cells acquire mutations that cause the cancer stem cells (CSCs) to arise. The pool of CSCs accumulate further oncogenic mutations to cause formation of cancer. The primary tumour releases factors to form the pre-metastasis niche at distinct sites. In return, the cells from the niche release homing factors to guide circulating metastatic cancer stem cells (mCSCs) to their location. Once present, the metastasis niche can either proliferate to form a metastatic lesion or remain dormant awaiting reactivation to become a secondary tumour.

Figure reproduced by permission from Macmillan Publishers: Cell Research, Li et al. 2007 [55]
4.3 Chemotherapeutic resistance of SP cells

Consistent with studies that show ABCG2 to be a molecular determinant of the SP phenotype [38], expression of ABCG2 mRNA was markedly higher in SP for all lung cancer cell lines analyzed. Interestingly, the results also revealed that the SP had elevated levels of other members of the ABC transporter family including ABCA2, MDR1 and MRP1 (and related subfamily members MRP2 to 9 that are potential drug pumps), that are known to export different chemotherapeutic drugs and associate with drug resistance [56]. Given that stem cells often display higher tolerance to cytotoxins [50], it is reasonable that SP cells in lung cancer also turn on a number of multi-drug-resistance genes as protective mechanisms. In support of this, we found that SP cells showed increased resistance to multiple chemotherapeutic drugs, a number of which, notably cisplatin, gemcitabine and vinorelbine, are commonly used as first-line therapy for lung cancer. Because different ABC transporters show overlapping yet different substrate specificity, the combination of these likely accounts for the range of drug resistance observed in the SP.

The study has established that lung cancer SP cells are more tumourigenic than non-SP cells. Since the SP phenotype has been associated with the ABC transporters, it would be interesting to examine the relationship between tumourigenicity and ABC transporter expression. A study by Patrawala et al. examined SP and non-SP in several cancer cell lines also investigated tumourigenic potential of ABCG2+ and ABCG2− cells [46]. Much to the surprise of the authors, the results revealed no significance difference between the two populations. Furthermore, they suggested that the ABCG2− population contained
more primitive stem-like cancer cells as shown in their proliferation and self-renewal assays. They reasoned this was attributable to the heterogeneous feature of the SP, as mentioned previously, and the possible existence several other subpopulations of cells that express different ABC transporters in addition the ABCG2\textsuperscript{+} cells, similar to the results of my project. The importance of dismissing ABCG2 as an indicator of tumourigenicity is a reminder of the difficult task in finding a specific marker that identifies the tumour-initiating cell. One must look for a combination of potential markers to establish an expression signature that may isolate this cell.

4.4 hTERT, MCM7, BMI1, Notch

This study found that expression levels of hTERT was elevated in SP cells from all cell lines tested. This is consistent with the work of Alvi and colleagues that showed elevated hTERT level in the SP of normal mammary epithelium [57]. In lung cancer, telomerase is expressed early in the multistage process and has been implicated in malignant transformation and tumour invasion. Furthermore, telomerase is a crucial marker of cellular immortalization in cancers [58]. The elevated expression of hTERT further indicates an enrichment of stem-like lung cancer cells in the SP. According to the cancer stem cell model, cancers likely have a subpopulation with indefinite repopulation potential. Thus, with its increased hTERT expression, the SP may represent such a reservoir for generating cancer cells, driving cancer cell immortalization and disease progression. As proposed by previous studies, this telomerase expression in cancer stem cells may be inherited from their normal stem cell counterpart, and is progressively lost during differentiation and maturation [32, 59].
The expression of MCM7, one of the members of the mini-chromosome maintenance family of proteins, was lower in the SP fraction, suggesting that SP cells are mainly outside of the active cell cycle. This is consistent with the concept that stem cells are mostly in the quiescent state [50]. MCMs are vital for DNA replication, thus are expressed higher during proliferation. Stem cells are known to be more quiescent, therefore the lower expression of MCMs in cells of the SP fraction further confirmed that SP are enriched with cancer stem cells. Interesting, BMI-1 expression was elevated in SP compared to the non-SP in four for the six cell lines tested. Also, higher expression of Notch-1 was detected in the SP compared to the non-SP of five of the six cell lines. Both genes are part of signalling pathways involved with self-renewal and cancer proliferation. Studies have identified proteins of the Notch-1 self-renewal pathways in SP [46]. It has been shown that expression of BMI-1 is required for proliferation and self-renewal of cerebellar progenitor cells [60]. Stem cells accumulate mutations that inappropriately cause higher activation Notch-1 and BMI-1 pathways and potentially forming cancer stem cells that lead to cancer. This reinforces the likelihood that SP in lung cancer is enriched with cancer stem cells.

4.5 Stem cell associated markers

The inability to determine stem cell surface markers for lung cancer established the importance of SP as method of isolating cancer stem cells. It exemplified the specificity of stem cells markers associated with each tissue, and the difficulty to elucidate a marker unique to lung cancer stem cells. The markers CD23, CD34, CD44 and Nestin, while chosen for their effectiveness as stem cell markers for other tissues, were only a few possible potential candidates within a vast pool of cell surface markers that was tested.
Therefore, the possibility of one or several of surface markers for lung stem cells to exist is highly likely. The use of SP on lung cancer is the first step in isolating the putative lung cancer stem cell and can act as a platform in which specific cell surface markers can be identified. Once discovered, these putative markers may provide a unique marker expression signature and allow us to isolate a more refined population of lung cancer stem cells.

4.6 SP in clinical lung tumour resections
This study reports the existence of a similar small SP fraction, as defined by the Hoechst 33342 dye efflux assay, in 16 human clinical lung cancer samples. It is possible that these SP cells also exhibit characteristics of a tumour-initiating, cancer stem cell phenotype. The presence of such a population with both high tumourigenic potential and drug resistance can have important clinical implications in lung cancer treatment. These rare cells have the potential to survive conventional chemotherapy and regenerate a cancer population, leading to relapse. Hence the SP may represent both a useful predictor of treatment response and target for effective treatment. Further studies may be needed to confirm the stem cell potential of SP in clinical lung tumour specimens, such in-vivo tumourigenicity tests and expression of stemness genes.
4.7 Summary and future goals
My study was the first to define and isolate an enriched tumour-initiating population in lung cancer. Using the SP phenotype, I was able to isolate and characterise SP cells in lung cancer cell lines that were significantly enriched for tumourigenicity and invasiveness. The SP cells also possess stem cell properties of multi-drug resistance, high telomerase activity, repopulating capacity, and quiescence. Although the SP does not necessarily represent 100% pure cancer stem cells or all of the malignant stem cells from the whole population, it is a significant enrichment of these rare cells responsible for initiating and maintaining cancer.

One of the most difficult tasks involved in isolating cancer stem cells is the lack of universally accepted markers. The potential stem cell markers tested in this study (CD24, CD34, CD44 and nestin) did not associate with Hoechst-dim cells, therefore cannot replace the Hoechst 33342 efflux assay in isolating lung cancer stem-like cells. However, there are also other potential markers that can be tested, as each stem cell marker is highly tissue-specific. For now, the distinct SP phenotype provides an attractive testing model for studying lung cancer-initiating cell biology and a framework for testing potential lung cancer stem cell markers.

Future work could consist of resolving the limitations of the SP approach to fully optimize the use of this method. As SP does not represent the entire stem cell population, modifications to this technique are required to improve the enrichment process. The finding of molecular markers for lung stem cells will be needed to complement the SP method. Another drawback to the SP method was the toxicity of the Hoechst 33342 dye
on cells. It is accepted that the concentration of the dye used in most SP studies are below levels that will cause cell death, but additional studies are necessary to optimize and define the conditions associated with minimum toxicity for lung tissue. Furthermore, the use of Hoechst dye to isolate SP cells could be avoided if a specific marker for SP is found. A recent study discovered CD55 as a novel marker for SP cells in breast carcinoma cell lines [61]. The authors were able to first use SP to determine the expression of the new marker to isolate SP cells then proceeded to isolate SP cells by CD55+ expression to perform downstream experiments, thus avoiding drug-associated cell toxicity.

While there are many similarities between the molecular profiles of normal and cancer stem cells, there are differences as well. As mentioned before, BMI-1 has an important role in self-renewal for both normal haematopoietic and leukemia stem cells. On the contrary, leukemic stem cells lose expression of the PTEN tumour suppressor, which functionally distinguishes them from normal haematopoietic stem cells that expressed this protein. Therefore future work would involve identifying and characterising unique molecular profile of cancer stem cells that discriminate them from normal stem cells.

My study also established the existence of SP in clinical lung resections. Identification of SP in clinical samples provides the evidence for the cancer stem cell within lung tumours. Future work could involve characterising the SP from these samples, such as examining tumourigenicity between SP and non-SP cells using mouse models. If SP in lung tumours are similar to the SP in lung cancer cell lines in terms of their increased tumour-
initiating ability, the importance of targeting these cells during therapy will be prominent.

It would be beneficial to examine the correlation between the existence of SP and resistance to chemotherapy in lung cancer patients. Other future goals could involve examining the gene expression of SP and non-SP cells isolated from lung tumours. A comparative genomic hybridization study to examine for genetic differences between SP and non-SP cells within a lung tumour can reveal different molecular expression characteristics and identify lung cancer stem cell associated genetic markers. The existence of SP in clinical lung resections provides the opportunity for future work in potentially identifying important targets for therapy.
5.0 REFERENCES


6.0 APPENDICES

6.1 Publications

Journal Articles:
*Both authors contributed equally to this work and are considered co-first authors.

Conference Abstracts:

Alvin V. Ng, Maria M. Ho, Jaclyn Y. Hung. Characterization of a side population, a subpopulation of cells enriched for cancer “stem” like activity in lung. British Columbia Cancer Agency Annual Cancer Conference 2006, Vancouver, Canada, November 23rd to 25th, 2006

Maria M. Ho, Alvin V. Ng, Jaclyn Y. Hung. Side population in human lung cancer cell lines and tumors is enriched with stem-like cancer cells. The First Japan Cancer Association – American Association of Cancer Research Special Joint Conference, Nagoya, Japan, March 12th to 14th, 2007


Alvin V. Ng, Maria M. Ho, Jaclyn Y. Hung. Side population in human lung cancer cell lines and tumors is enriched with stem-like cancer cells. Pathology Day, University of British Columbia, Vancouver, British Columbia, Canada, May 24th, 2007
6.2 Statement of work

The project was completed by Maria Ho and I under the guidance of Dr. Jaclyn Hung. I performed the majority of the cell culture work and the side population analysis, including the Hoechst dye efflux assay. The FACS experiments were performed together with technicians at Terry Fox Lab. mRNA extraction and real-time RT-PCR experiments was initially performed by Maria (ABC transporters). After learning the technique from Maria and Jaclyn, I designed the primers of other stemness genes and analyzed their expression. Tumour cell implantation experiments were performed together in the Animal Research Facility. I was entirely responsible for re-analysis of SP-derived tumours, invasion experiments, drug sensitivity experiments, and the clinical lung tumour resection analyses. Multiple cell-surface marker staining was performed by both Maria and I.