P-2628

PHENOTYPIC CHARACTERIZATION OF SIDE POPULATION IN BREAST CANCER CELL LINE MCF7

A Project Report

Submitted by

CHRISTINA ANN KURIAN

In partial fulfillment for the award of the degree of

BACHELOR OF TECHNOLOGY

IN

BIOTECHNOLOGY

KUMARAGURU COLLEGE OF TECHNOLOGY

ANNA UNIVERSITY::CHENNAI 600 025

APRIL 2009

ANNA UNIVERSITY : CHENNAI 600 025

BONAFIDE CERTIFICATE

Certified that this project report **"PHENOTYPIC CHARACTERIZATION OF SIDE POPULATION IN BREAST CANCER CELL LINE MCF7"** is a bonafide work of **"CHRISTINA ANN KURIAN"** who carried out the project work under my supervision.

SIGNATURE 27/4/09

DR. P. RAJASEKARAN

HEAD OF DEPARTMENT
DEPARTMENT OF BIOTECHNOLOGY
KUMARAGURU COLLEGE OF TECHNOLOGY
CHINNAVEDAMPATTI
COIMBATORE-641006

24/04/09

SIGNATURE

DR. K. KUMARESAN

(SUPERVISOR) SENIOR LECTURER
DEPARTMENT OF BIOTECHNOLOGY
KUMARAGURU COLLEGE OF TECHNOLOGY
CHINNAVEDAMPATTI
COIMBATORE-641006

RAJIV GANDHI CENTRE FOR BIOTECHNOLOGY

A CENTRE FOR ADVANCED RESEARCH AND TRAINING IN BIOTECHNOLOGY

No.35/C/RGCB/2009
March 25, 2009

CERTIFICATE

This is to certify that this project entitled **"Phenotypic Characterization of Side Population in Breast Cancer Cell Line – MCF7"** is a bonafide record of project work done by **Ms. Christina Ann Kurian** under my supervision and guidance from January 10, 2009 to April 10, 2009 in partial fulfillment of the requirement for the award of the degree of Bachelor of Technology in Biotechnology from Anna University.

Professor M. Radhakrishna Pillai FRCPath, Ph D, FASc, FNASc
DIRECTOR

Professor M. RADHAKRISHNA PILLAI
FRCPath, PhD, FASc, FNASc
DIRECTOR
RAJIV GANDHI CENTRE FOR BIOTECHNOLOGY
THIRUVANANTHAPURAM - 695 014, INDIA

CERTIFICATE OF EVALUATION

COLLEGE : Kumaraguru College of Technology

BRANCH : Industrial Biotechnology

SEMESTER : Eight Semester

NAME OF STUDENT	TITLE OF PROJECT	NAME OF SUPERVISOR
Christina Ann Kurian (71205214005)	**PHENOTYPIC CHARACTERIZATION OF SIDE POPULATION IN CANCER CELL LINE MCF7**	**Dr. K. Kumaresan (Senior Lecturer)**

The report of the project work submitted by the above student in partial fulfillment for the award of Bachelor of Technology degree in Biotechnology of Anna University was evaluated and confirmed to be the report of the work done by the above student. It was submitted for evaluation and viva-voce held on 27-4-2009

INTERNAL EXAMINER 27/4/09

EXTERNAL EXAMINER

ACKNOWLEDGEMENT

I express my deep sense of gratitude to Dr. P. Rajasekaran, Head of the Department, Department of Biotechnology, Kumaraguru College of Technology, Coimbatore for all the guidance and help in all ways throughout the project work.

I would like to express my sincere thanks to my guide Dr. M. Radhakrishna Pillai, Director, Ragiv Gandhi Centre of Biotechnology for his unfailing guidance and support throughout the work and thereafter.

I would like to acknowledge the support and help given by Ms. Shanti Achuthan, Junior Research Fellow, Ragiv Gandhi Centre of Biotechnology. Furthermore I express my gratefulness to the institute for lending me their time, space and resources.

My heartfelt gratitude to my internal guide Dr. K. Kumaresan, Senior Lecturer, Department of Biotechnology, Kumaraguru College of Technology, Coimbatore, for his valuable proposition and analysis.

I would like to acknowledge thanks to my parents, friends and classmates for their timely help and moral support. Finally I remember the kind help from all the non-teaching members of the Department of Biotechnology.

Christina Kurian

Christina Ann Kurian

ABSTRACT

Cancer is a disease wherein a group of cells show uncontrolled growth. It is a genetic disease that alters three types of genes which are responsible for tumor progression, namely oncogenes, tumor suppressor genes and stability genes. The reoccurrence of breast cancer has been high in the recent and is it hypothesized that this maybe because of a small population of cells that escape the treatment. Stem cells are those cells which have very efficient membrane pumps and enable them to protect themselves from influx of harmful chemicals. "Side Population" (SP) cells, which pump out the fluorescent dye Hoechst 33342 via the ABCG2 transporter, define a putative stem/progenitor cell population. Breast cancer SP cells has been isolated from the MCF-7 cell line which possess similar properties and may represent stem cell-like cancer cells which are found to have very high tumorigenicity and has to ability to form colonies. 1.1% of side population was isolated from MCF7 and immunophenotyping showed that these side population cells are highly enriched for Multi Drug Resistant (MDR) markers.

TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

1. INTRODUCTION

Breast cancer is the most common cause of cancer in women and the fifth most common cause of cancer death in the world. Although breast cancer in women is a common form of cancer, male breast cancer too does occur.

Breasts are found on the chest muscles at the ribs. They contain 15-20 lobes. Each lobe is made up of lobules which consist of glands responsible for milk secretion. The milk is secreted from the lobules to the nipple by tubes called ducts. The nipple leads to the centre of a dark-skinned area called the areola. Fat fills the space between the lobules and the ducts. Breasts also contain lymph vessels which lead to small round organs called lymph nodes which are found near the breasts in the axilla (armpit), above the collar bone, on the chest behind the breast bone and in many other parts of the body. Lymph nodes have the tendency to trap harmful substances like bacteria and cancer cells (Fig 1.1).

The breast mainly consists of two types of epithelial cells: myoepithelial cells and luminal epithelial cells. The breasts rest on a muscle called pectoralis major that overlays the ribcage and are supported by suspensory ligaments and contain varying amounts of fatty tissue.

Breast cancer originates in the ducts and tubules in the breast. When breast cancer cells spread, they are found in the lymph nodes near the breast. They also spread to other parts of the body likes bones, liver, lungs and brain. This spreading of cancer cells to distant locations is achieved through metastasis.

Breast cancer occurs more commonly in the left breast than the right and more commonly in the right upper quadrant. The tumor can change the shape of the breast and the texture of the skin.

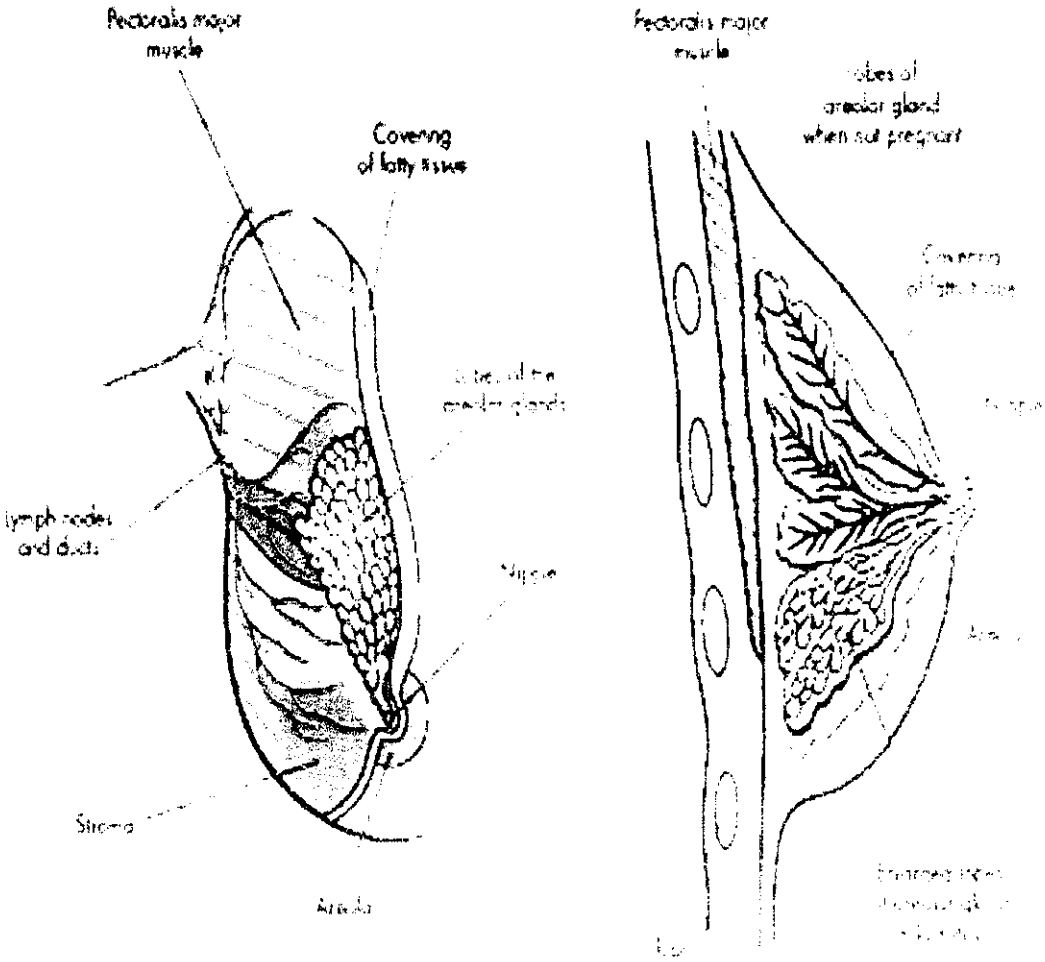


Fig 1.1 The Human Breast

1.1 CANCER PROCESS

Cancer begins in cells; cells grow and divide to form new cells as the body needs them. When cells grow old, they die, and new cells take their place. Sometimes, this orderly process goes wrong. New cells form when the body does not need them, and old cells do not die when they should. These extra cells can form a mass of tissue called a growth or tumor (Fig 1.2).

Tumors can be benign or malignant:

Benign tumors are not cancer. Benign tumors are rarely life-threatening and it can be removed. Cells from benign tumors do not invade the tissues around them. Cells from benign tumors do not spread to other parts of the body.

Malignant tumors are cancer. Malignant tumors are generally more serious than benign tumors. They may be life-threatening. Cells from malignant tumors can invade and damage nearby tissues and organs. Cancer cells spread by breaking away from the original (primary) tumor and entering the bloodstream or lymphatic system. The cells invade other organs and form new tumors that damage these organs. The spread of cancer is called metastasis. Breast cancer can spread to almost any other part of the body. The new tumor has the same kind of abnormal cells and the same name as the primary tumor.

Based on the tumour, the histological appearance and location of the lesion, the breast cancer can be classified as follows:

- Adenocarcinoma : arising from the epithelium
- Intraductal : developing within the ducts
- Infiltrating : occurring in parenchyma of the breast
- Inflammatory : rapid tumour growth occurs in which the overlaying skin becomes oedematous.

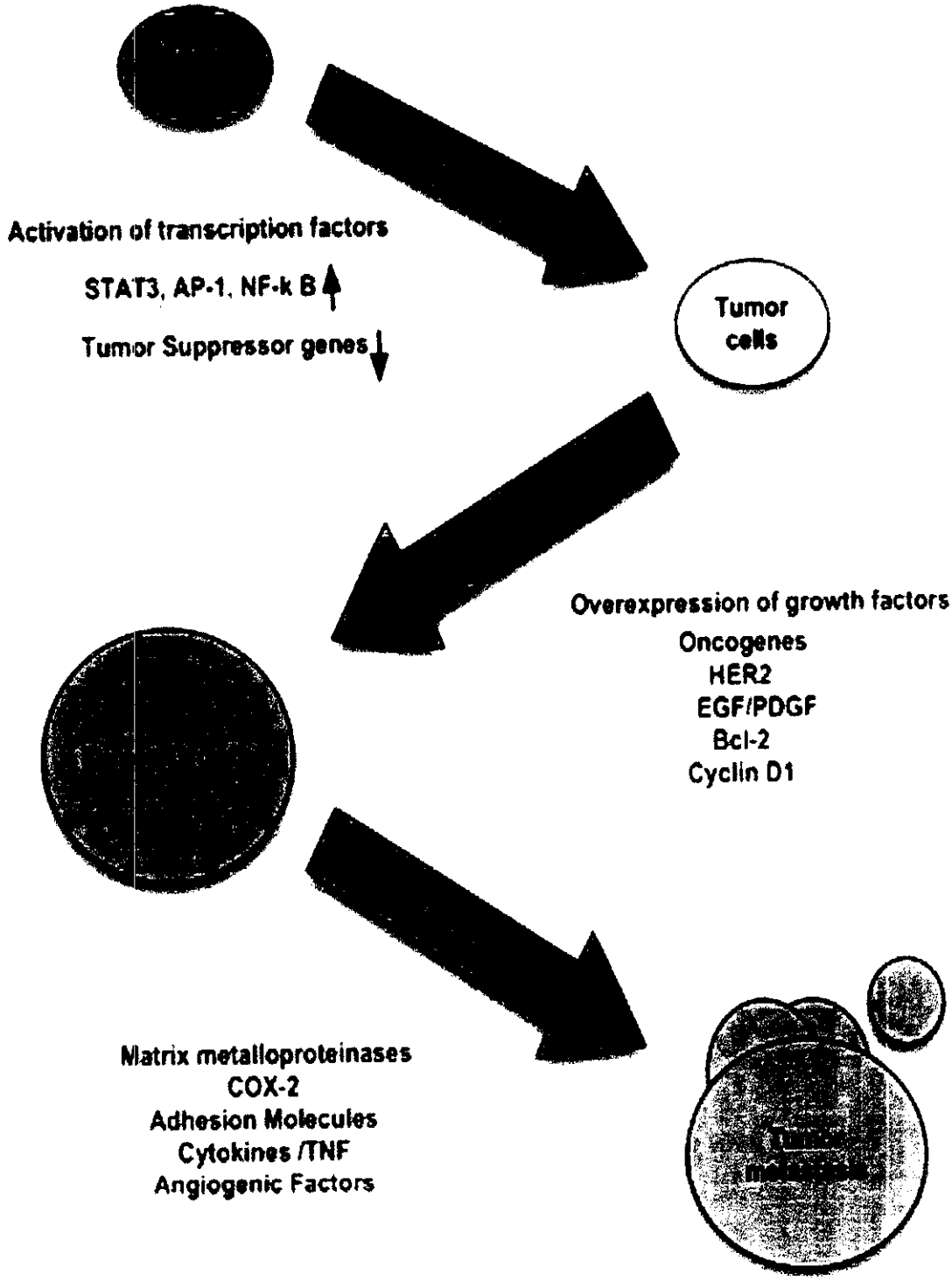


Fig 1.2 Tumourigenesis

1.2 CAUSE

No one knows the exact causes of breast cancer. A risk factor is something that may increase the chance of developing a disease. Studies have found the following risk factors for breast cancer:

Family history: A woman's risk of breast cancer is higher if her mother, sister, or daughter had breast cancer.

Gene changes: Changes in certain genes increase the risk of breast cancer. These genes include BRCA1, BRCA2, and others. Of which mutation in BRCA1 is the main cause of cancer in women. Tests can sometimes show the presence of specific gene changes in families with many women who have had breast cancer.

Reproductive and menstrual history: The older a woman is when she has her first child, the greater her chance of breast cancer. Women who had their first menstrual period before age 12 and those who went through menopause after age 55 are at an increased risk of breast cancer. Women who never had children and women who take menopausal hormone therapy with estrogen plus progestin after menopause also appear to have an increased risk of breast cancer.

Radiation therapy to the chest: Women who had radiation therapy to the chest (including breasts) before age 30 are at an increased risk of breast cancer. Studies show that the younger a woman was when she received radiation treatment, the higher her risk of breast cancer later in life.

Lifestyle: The chance of getting breast cancer is higher in women who are overweight or obese and those who are physically inactive throughout life. Studies suggest that the more alcohol a woman drinks, the greater her risk of breast cancer.

1.3 SYMPTOMS

Common symptoms of breast cancer include:

- A lump or thickening in or near the breast or in the underarm area.
- Nipple tenderness or discharge
- A change in the size or shape of the breast
- A nipple turned inward into the breast
- The skin of the breast, areola, or nipple may be scaly, red, or swollen. It may have ridges or pitting so that it looks like the skin of an orange.

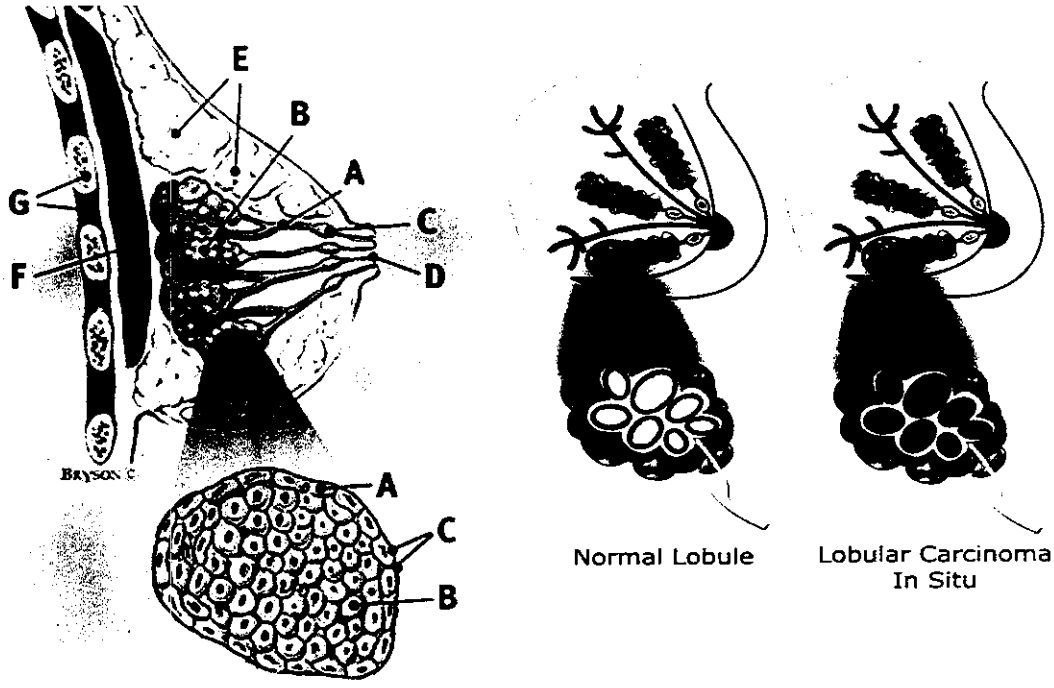
1.4 STAGING

The stage is based on the size of the tumor and whether the cancer has spread. Staging may involve x-rays and lab tests. These tests can show whether the cancer has spread and, if so, to what parts of your body. Given below are the different stages of breast cancer:

1.4.1 Stage 0

Lobular carcinoma in situ (LCIS): Abnormal cells are in the lining of a lobule. LCIS seldom becomes invasive cancer. However, having LCIS in one breast increases the risk of cancer for both breasts (Fig 1.3).

Ductal carcinoma in situ (DCIS): Abnormal cells are in the lining of a duct. DCIS is also called intraductal carcinoma. The abnormal cells have not spread outside the duct. They have not invaded the nearby breast tissue. DCIS sometimes becomes invasive cancer if not treated (Fig 1.4).



A-Ducts

B-Lobules

C-Dialated Section of Duct to Hold Milk

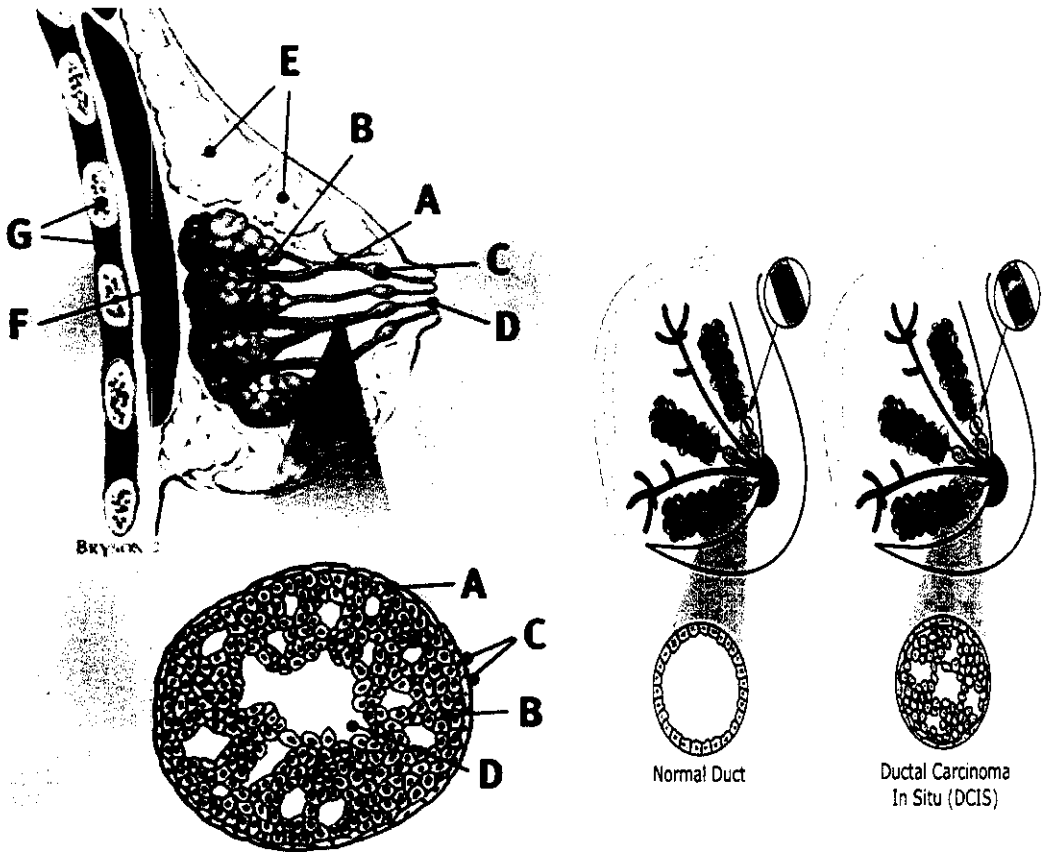
D-Nipple

E-Fat

F-Pectoralis Major Muscle

G-Chest Wall or Rib Cage

Fig 1.3 Lobular Carcinoma In Situ



A-Ducts

B-Lobules

C-Dilated Section of Duct to Hold Milk

D-Nipple

E-Fat

F-Pectoralis Major Muscle

G-Chest Wall or Rib Cage

Fig 1.4 Ductal Carcinoma In Situ

Two categories of DCIS are present:

- Comedo type
- Non comedo type

Comedo type: The term comedo describes the appearance of the cancer. When comedo type breast tumours are cut, the dead cells inside them (necrosis) can be expressed out just like a comedo or black head on the skin. These types of DCIS are more aggressive than the non comedo type of DCIS (Fig 1.5).

Non comedo type: Most common non comedo types of DCIS are

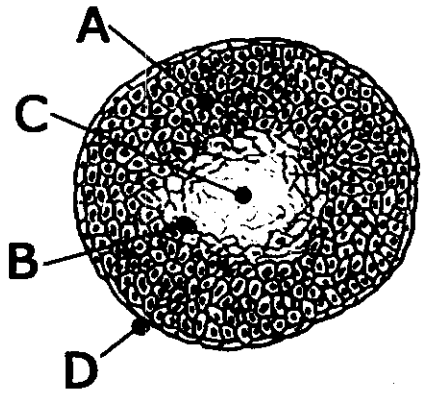
- Solid DCIS: Cancer cells completely fill the affected breast ducts
- Cribiform DCIS: Cancer cells do not completely fill the affected breast ducts
- Papillary and micro papillary DCIS: Cancer cells arrange themselves in a fern like pattern within the affected breast ducts (Fig 1.6).

1.4.2 Stage I

It is an early stage of invasive breast cancer. The tumor is no more than 2 centimeters across. Cancer cells have not spread beyond the breast.

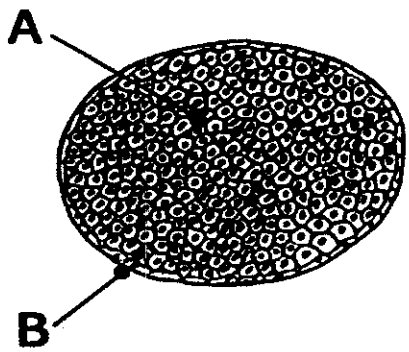
1.4.3 Stage II

The tumor in the breast is no more than 2 centimeters across and has spread to the lymph nodes under the arm. The tumor is between 2 and 5 centimeters and may have spread to the lymph nodes under the arm. The tumor is larger than 5 centimeters but has not spread to the lymph nodes under the arm.

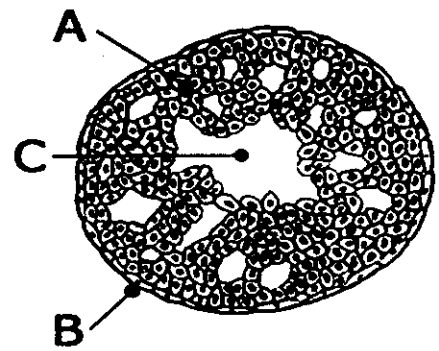


A-Living Cancer Cells
B-Dying Cancer Cells
C-Cell Debris (Necrosis)
D-Basement Membrane

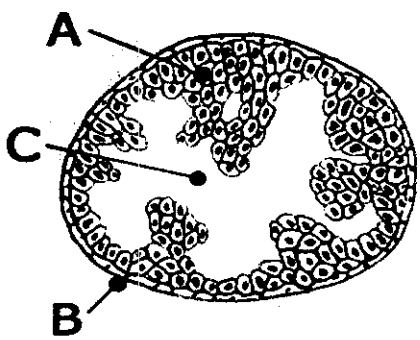
Fig 1.5 Comedo Type



Solid Dcis



Cribiform Dcis



Papillary Dcis

Fig 1.6 Non Comedo Type

1.4.4 Stage III

This is locally advanced cancer. It is divided into Stage IIIA, IIIB, and IIIC.

Stage III A: The tumor in the breast is smaller than or greater than 5 centimeters. The cancer has spread to underarm lymph nodes that are attached to each other or behind the breastbone

Stage IIIB: The tumor may be associated with swelling of the breast or with nodules in the breast skin. The cancer may have spread to underarm lymph nodes that are attached to each other or the lymph nodes behind the breastbone. *Inflammatory breast cancer* is a rare type of breast cancer. The breast looks red and swollen because cancer cells block the lymph vessels in the skin of the breast.

Stage IIIC: The cancer has spread to the lymph nodes behind the breastbone and under the arm or the cancer has spread to the lymph nodes under or above the collarbone.

1.4.5 Stage IV

It is distant metastatic cancer. The cancer has spread to other parts of the body. Breast cancer has spread beyond the breast and nearby lymph nodes, even though this is the first diagnosis of breast cancer. The reason for this is that the primary breast cancer was not found when it was only inside the breast. Metastatic cancer is considered stage IV.

1.5 TREATMENT

Breast cancer treatment is based on certain factors which include: Tumor size, tumor type, histological grade, lymph node status, distant metastatic cancer, Estrogen or progesterone receptors, Her-2-neu receptors etc.

Women with breast cancer have many treatment options. These include surgery, radiation therapy, chemotherapy, hormone therapy, target therapy and biological therapy. Many women receive more than one type of treatment.

Cancer treatment is either local therapy or systemic therapy:

1. Local therapy: Surgery and radiation therapy are local treatments. They remove or destroy cancer in the breast. Local therapy may be used to control the disease in those specific areas.

- **Radiation therapy**: It uses high energy rays to kill cancer cells. Depending upon the type of the tumor and other factors, radiation may be given. Radiation destroys breast cancer cells that may remain in the area.

- **Surgery**: This is the most common treatment for breast cancer and there are several types of surgery: breast conserving surgery, lumpectomy, segmental mastectomy or partial mastectomy and mastectomy.

2. Systemic therapy: Chemotherapy, hormone therapy, and biological therapy are systemic treatments. They enter the bloodstream and destroy or control cancer throughout the body. Systemic treatments also are used for cancer that has spread.

- **Chemotherapy**: It is usually a combination of anticancer drugs to kill cancer cells. The drugs may be given as a pill or by injection in to the vein. Eg: Doxorubicin, Taxol.

- **Hormonal therapy**: blocks certain hormones that enhance cancer growth. There are certain hormones that can attach to cancer cells and can affect their ability to multiply. The purpose of hormone therapy is to add, block or remove hormones. Eg: Tamoxifen.

  There are two types of hormone therapy for breast cancer:

  - Drugs that inhibit estrogen and progesterone from promoting breast cancer cell growth.
  - Drugs or surgery to turn off the production of hormones from the ovaries.

Recurrent breast cancer

Recurrent cancer is cancer that has come back after it could not be detected. Treatment for the recurrent disease depends mainly on the location and extent of the cancer. Another main factor is the type of treatment the woman had before.

If breast cancer comes back only in the breast after breast-sparing surgery, the woman may have a mastectomy. Chances are good that the disease will not come back again.

If breast cancer recurs in other parts of the body, treatment may involve chemotherapy, hormone therapy, or biological therapy. Radiation therapy may help control cancer that recurs in the chest muscles or in certain other areas of the body.

Treatment can seldom cure cancer that recurs outside the breast. Supportive care is often an important part of the treatment plan. Many patients have supportive care to ease their symptoms and anticancer treatments to slow the progress of the disease. Some receive only supportive care to improve their quality of life.

OBJECTIVES

2. OBJECTIVES

1. To identify the presence of Side Population cells from the breast cancer cell line MCF7.
2. To sort the Side Population cells from the breast cancer cell line MCF7.
3. To find the different MDR (Multi Drug Resistant) markers present in the sorted Side Population cells.

LITERATURE REVIEW

3. LITERATURE REVIEW

Stem cells have the remarkable potential to develop into many different cell types in the body. Serving as a sort of repair system for the body, they can theoretically divide without limit to replenish other cells as long as the person or animal is still alive. When a stem cell divides, each new cell has the potential to either remain a stem cell or become another type of cell with a more specialized function, such as a muscle cell, a red blood cell, or a brain cell. (The National Institute of Health).

In 1998 cells were successfully removed from spare embryo at fertility clinic and grew them in a laboratory. This launched the stem cell research into lime light, establishing the world's first human embryonic cell line which still exists today. (UK Stem Cell Foundation).

Stem cells are those cells which have the unique ability for self renewal and to perform the maintenance of their tissue of origin. The stem cells are important in sustaining long term repopulation of the specific tissue via progression through a series of increasingly differentiated cells. There already exists evidence from animal studies that stem cells can be made to differentiate into cells of choice, and that these cells will act properly in their transplanted environment. (Terumasa *et al.*, 2008)

The cancer stem cell hypothesis was first documented in acute myelogenous leukemia (AML) by John in 1997. These authors demonstrated that only a minority of the leukemic cells were able to proliferate extensively, self-renew and form a new tumor.

Cancer stem cells are a small population of tumor cells capable of self-renewal that give rise to all the components of a heterogeneous tumor. In previous models of cancer, the unregulated growth of tumors was attributed to the serial acquisition of genetic events that resulted in the activation of genes promoting proliferation, silencing of genes involved in inhibiting proliferation, and circumventing genes involved in programmed cell death. (Al-Hajj *et al.*, 2003)

In the cancer stem cell hypothesis, another key event in tumor progression is the alterations of genes involved in the regulation of stem cell renewal. Thus, it is not surprising that several genes initially identified due to their role in tumor progression were later implicated in normal stem cell self-renewal. (Karen *et al.*, 2005)

Definite evidence was brought on the importance of cancer stem cells in the progression of cancer. Key markers of there cells have been identified in many solid tumors. Specific studies modeling the tumor induction of these cells have demonstrated that they are key units in their tumorigenecity. (Kevin *et al.*, 2007)

Studies have provided evidence for the existence of mammary stem cells in mice, rats and humans. Subsequent identification and isolation of these stem cells has shown their ability to repopulate cleared mammary fat pads and give rise to all the structures seen in normal mature glands. (Coppock and Clarke 2004)

The phenotypic and functional differences between cells that initiate human breast tumors (cancer stem cells) and those that comprise the tumor bulk are difficult to study using only primary tumor tissue. It was embarked on this study hypothesizing that breast cancer cell lines would contain analogous hierarchical differentiation programs to those found in primary breast tumors. These data validate the use of cancer cell lines as models for the development and testing of novel therapeutics aimed at eradicating cancer stem cells. (Christine and Kuperwasser 2008)

Side population cells are a rare subset of cells found in various tissues that are highly enriched for stem cell activity and showed that these are a special population of cells that possesses most of the stem cell characteristics. These populations of cells are called as side population cells because; they are very small in number when compared to main population of cells. (Ross *et al.*, 2003)

In this glioma model, CSCs are enriched in the side population (SP) cells. These SP cells have enhanced tumor-initiating capacity, self-renewal, and multi potentiality compared with non-SP cells from the same tumors. (Molly *et al.*, 2008)

Side population cells are a rare subset of cells found in a variety of tissues with the unique ability to efflux lipophilic dyes such as Hoechst 33342. They are highly enriched for different types of membrane transporters with the help of which, SP cells are stained low by dyes and this low staining character of SP cells is utilized to identify them from other main population cells.

Isolation of side population cells from murine bone marrow cells was carried out with the help of vital dye Hoechst 33342. Competitive repopulation experiments showed that, they are enriched for HSC activity and displays in vivo reconstitution activity. They showed that these cells were able to protect recipients from lethal irradiation at low cell doses. These populations of cells are called as side population cells because; they are very small in number when compared to main population of cells (Goodell *et al.*, 1996)

Hoechst 33342 is a DNA binding fluorescent dye that binds perpendicularly to A-T rich regions of DNA and the dye is excited by UV wavelengths (maximum excitation is 395nm) and emits in the blue region (emission maximum is 450nm).

Flow cytometry and the DNA-binding dye Hoechst 33342 to isolate SP cells from various human gastrointestinal system cancer cell lines. Fifteen of sixteen cancer cell lines from the gastrointestinal system contained0.3%–2.2% SP cells. Next, we used an oligonucleotide microarray to analyze differentially expressed genes between SP and non-SP cells. (Naotsugu *et al.*, 2006)

It was found out that mammary gland contains a distinct population of Hoechst effluxing side population cells called as mammary gland side population cells (MG SP). They did transcriptional profiling and suggested that MG SPs are a lineage deficient

mammary gland sub population, which expresses key genes involved in cell cycle regulation development and angiogenesis (Fariba *et al.*, 2006).

Many cancer cell lines contain a small side population (SP), which, in many normal tissues, is thought to contain the stem cells of the tissue. It was demonstrated that in the absence of serum, the combination of basic fibroblast growth factor and platelet-derived growth factor maintains SP cells in the C6 glioma cell line. Moreover, C6 SP cells, but not non-SP cells, can generate both SP and non-SP cells in culture and are largely responsible for the in vivo malignancy of this cell line. Finally, evidence was provided that C6 SP cells can produce both neurons and glial cells in vitro and in vivo. It was proposed that many cancer cell lines contain a minor sub population of stem cells that is enriched in an SP, can be maintained indefinitely in culture, and is crucial for their malignancy. (Toru *et al.*, 2004)

Experiments in thyroid cancer cell lines showed the presence of SP cells that contain cancer stem cells. In addition, they showed that, the sorted SP cells have the capacity to generate both SP and non-SP cells in culture and the clonogenic ability of SP cells was significantly higher than that of non-SP cells. (Norisato *et al.*, 2007)

Isolation and characterization of SP and non-SP population from normal and malignant human kidney tissue have been carried out. They showed that the normal renal epithelial cells constituted about 3.8%SP cells and the malignant renal cells constituted about 5.9% of SP cells. It was also revealed that, these renal SP is enriched for quiescent cells, with a high proliferative capacity and stem like properties. (Sanjai *et al.*,2008)

Studies of mouse and human breast SP suggest that the population is undifferentiated but capable of differentiating into epithelial structures of both luminal and myoepithelial lineages both *in vitro* and *in vivo*. Evidence that the SP is enriched for stem cells is only correlative, and there are potentially confounding technical issues. (Matthew and Clarke 2005)

Isolation of cardiac SP cells from neonatal rat hearts (CSP) showed that when green fluorescent protein positive CSPs were intravenously infused in to adult rats, some CSPs were migrated to injured heart than normal heart and this CSP in injured heart differentiated in to cardiomyocytes, endothelial cells or smooth muscle cells. Their results suggested that CSPs are intrinsic cardiac stem cells and involved in the regeneration of diseased hearts. They found out the expression of BCRP1 gene on the cell surface of CSP as well as bone marrow SP cells but not in MP cells. (Oyama *et al.*, 2005)

Isolation of side population cells from adult rat kidney and investigation of their differentiation potentials was performed. Analysis of kidney derived SP cells after bone marrow transplantation showed that some of the cells were derived from bone marrow. When enhanced green fluorescent protein (EGFP) labelled kidney derived SP cells were intravenously transplanted to wild type adult rats, EGFP+ cells in kidney could not be found but EGFP+ skeletal muscle, EGFP+ hepatocytes and EGFP+ bone marrow cells were observed. Thus they concluded that SP cells in kidney may have potentials for hematopoietic and non hematopoietic lineages but are not stem cells for renal cells. (Iwatani *et al.*, 2004)

For the first time, isolation of side population cells from nasopharyngeal carcinoma cells (NPC) was performed. Their results showed that SP cells in human NPC cell line CNE 2 had stem cell characteristics in vitro and they had a strong ability to form tumors in vivo. They found out the cell marker cytokine 19, which serves as a potential molecular marker for further characterization of cancer stem cell. (Wang *et al.*, 2007)

Identification of side population cells in xenografts and primary ovarian cancer samples were carried out and investigation on the effects of IFN-α on this tumor subset was done. This cytokine exerts marked anti proliferative and pro apoptotic effects on the SP subset than non-SP, which translate in to a therapeutic effect against tumors bearing large amounts of SP cells. (Lidia *et al.*, 2007)

It was demonstrated the existence of SP in human mammary epithelium and that purified SP are a single live cell population that have the ability to grow in vivo n in vitro. (Azra *et al.*, 2003)

It was shown that the SP cells can be isolated from a wide variety of mammalian tissues based on their dye efflux phenomenon and he showed that in many cases these SP cells contains multipotent stem cells(Grant and Little 2004)

The eukaryotic ABC genes are organized either as full transporters or as half transporters. Full transporters consists of two TMs and two NBFs. Half transporters consists of only one TM and one NBF and must combine with another half transporter to gain functionality.

There are more than 100 ABC transporters distributed from prokaryotes to humans. Moreover, phylogenetic analysis classifieds the 48 known human ABC transporters in to 7 distinct sub families of proteins. They are:

- **ABCA**-This family contains some of the largest transporters and five of them are located in a cluster in the 17q24 chromosome.
- **ABCB**- Consists of 4 full and 7 half transporters.
- **ABCC**- Consists of 12 full transporters.
- **ABCD**- Consists of 4 half transporters.
- **ABCE/ABCF**- Consists of 1 ABCE and 3 ABCF proteins.
- **ABCG**- Consists of 6 reverse half transporters, with the NBF at the NH3 + end and the TM at the COO- end.

Among these ABC transporters, members of ABCB1, ABCC1, and ABCG2 play a major role in multidrug resistance. Multi drug resistance is typically defined as the ability of a living cell to show resistance to a wide variety of structurally and functionally unrelated compounds. ABCB1 is otherwise known as p-glycoprotein and was encoded by the gene called MDR1 (Fig 3.1).

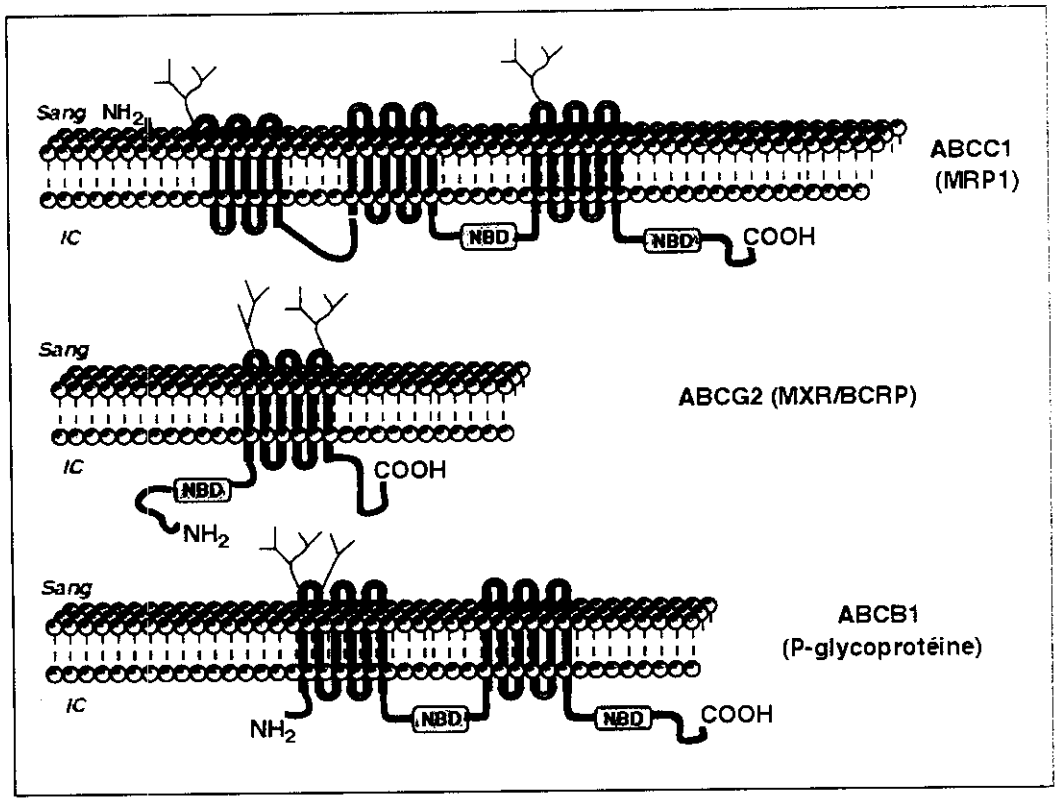


Fig 3.1 ABC Transporters

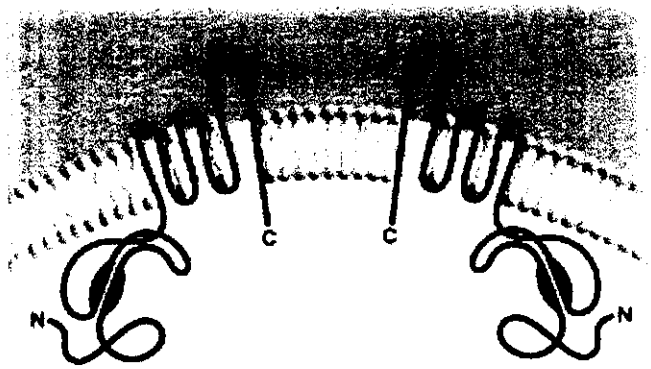


Fig 3.2 ABCG2 Trasnmembrane Transporters

It was reported that p-glycoprotein is a 170 k Da membrane protein glycosylated at extra cellular loop. It contains 12 hydrophobic transmembrane domains (TM) and 2 nucleotide-binding domains (NBD). (Cheol, 2005)

The three dimensional structures of p-glycoprotein and its conformational change in the presence and absence of nucleotide have been analyzed. These TMs will form a central pore that will help in the drug efflux. In the absence of a nucleotide, and upon binding of a nucleotide, the transmembrane domain will undergo reorganizations. This reorganization opens the central pore along its length in a manner that could allow access of hydrophobic drugs (transport substrates) directly from the lipid bilayer to the central pore of transporter. The known substrates of p-glycoprotein include natural product antineoplastics such as anthracyclines, vinca alkaloids, taxanes and epipodophyllotoxins. (Rosenberg *et al.*, 2001)

ABCB1 is also known as MRP1 or multidrug resistance related protein, which is a 190kDa protein that confers multidrug resistance on tumor cells by reducing drug accumulation by active efflux. It was first cloned in 1992 from a highly drug resistant lung cancer cell line-H69AR. It contains a core structure of two hydrophobic membrane-spanning domains, each followed by a nucleotide-binding domain. The two membrane spanning domains consists of 6 transmembrane α helices and in addition to this core, a NH2- proximal membrane spanning domain of 200 amino acids with an extracytosolic NH2 terminus was identified. MRP1 can actively transport a structurally diverse array of conjugated organic cations and it cannot transport unmodified chemotherapeutic agents.

Third, most important ABC transporter is ABCG2, which is also known as BCRP1- Breast Cancer Resistant Protein 1.This half transporter is located at chromosome 4q22 and it encodes a 655 amino acid polypeptide and consists of 6 putative transmembrane domains and one nucleotide binding domain (Fig 3.2).

It was reported that ABCG2 half transporter serves as a marker protein for side population selection. Comparison of mRNA levels for these three proteins; MDR1,

MRP1, and ABCG2 in bone marrow SP cells revealed that ABCG2 is the predominant form in these cells. (Zhou *et al.*, 2001)

Experiments to determine whether the stem cell effects required the efflux pump activity of p-glycoprotein or whether other proportions of the experimental system could cause the effects were carried out. A functionally inactive MDR1 mutant cDNA was used to show that p-glycoprotein pump function was required both for amplification of phenotypically defined SP cells and functionally defined repopulating cells. (Bunting *et al.*, 2000)

It was demonstrated that the technique of side population (SP) analysis used previously to identify haemopoietic stem cells [has been applied to the identification of mammary stem cells. SP cells are able to efflux the fluorescent dye, Hoechst 33342 by the ABCG2 transporter molecule, also known as Bcrp1. The SP fraction is enriched for stem cells, as it has been demonstrated that mouse mammary SP cells, which also expressed the mouse Sca-1, were able to repopulate cleared mammary fat pads of syngeneic hosts (Robert 2005)

It was found out that MCF7 SP cells had higher colony-formation ability *in vitro* and greater tumorigenicity *in vivo*, suggesting that MCF7 SP cells enrich cancer stem-like cells. cDNA microarray analysis of the SP cells indicated higher expression of ATP-binding cassette transporters (Jiangbing *et al.*, 2007)

Side population (SP) cells found in the MCF7 breast cancer cell line, were first confirmed by cell surface markers and gene profiling to be highly enriched in cells that fulfill specific functional, phenotypic, and molecular criteria for being tumor stem/progenitor cells. These cells express MUC1 and give rise to MUC1(+) tumors in vivo, which maintain the MUC1(+) SP population. MUC1 on SP cells is hypoglycosylated and heavily sialylated; the characteristics of the tumor-specific form were expressed on mature cancer cells and recognized by tumor-specific T cells and

antibodies. This suggests that stem/progenitor cells, like mature tumor cells, would be targets of MUC1-directed immunotherapy. (Katja *et al.*, 2008)

SP cells from mouse skin were identified using the same method as from bone marrow. This population almost completely disappeared after treatment with the calcium channel blocker verapamil. SP cells were mainly localized in the epidermis, with a few in the dermis. The ratio of SP cells decreased as the mouse became older. Surface marker analysis revealed that the sorted SP cells expressed α6-integrin, ß1-integrin, Sca-1, keratin 14, and keratin 19, which are proliferating and progenitor cell markers, at levels higher than in non-SP cells, while they expressed E-cadherin, CD34, and CD71 at lower levels. (Jihyun *et al.*, 2007)

SP cells was identified and isolated from gastro intestinal cancers. Experiments were carried out on hepatoma cell line HuH7 and colorectal SW480 cell lines and their results suggested a multi lineage potential for HuH7 SP cells and also these SP cells showed evidence for self renewal, generating both SP and non-SP cells. They also showed chemo resistance to anticancer drugs. (Naotsugu *et al.*, 2006)

Identification, isolation and characterization of side population cells in human tracheobronchial epithelium were performed. Using flow cytometry, CD45+, CD45- and NON SP cells were isolated and sorted. It was found that the number of CD45- SP cells was greater in the epithelium of asthmatic patients and they showed that all CD45- SP cells are enriched for epithelial markers such as cytokeratin 5, E- cadherin, ZO-1, p63 and CD45- SP cells exhibited stable telomere length and increased colony forming and proliferative potential. Finally, concluded that the number of SP cells is significantly greater in asthmatic airways. (Louise *et al.*, 2008)

The expression of breast cancer resistance protein 1 (BCRP1), which participates in dye efflux, was expressed at high levels at both the protein and mRNA level in sorted SP cells. (Shoichiro *et al.*, 2005)

The side population (SP) phenotype has been introduced as a reliable marker to identify subpopulations of cells with stem/progenitor cell properties in various tissues. ABCG2 belongs to the ATP-binding cassette (ABC) transporter super family and constitutes the molecular basis for the dye efflux, hence the SP phenotype, in hematopoietic stem cells. Another ABC transporter, MDR1, is the main contributor to the SP phenotype in the adult heart (Pfister *et al.*, 2008)

Observations on functional adenosine triphosphate (ATP)-dependent drug efflux in certain multidrug-resistant cancer cell lines without over expression of p-glycoprotein or multidrug resistance protein (MRP) family members suggested the existence of another ATP-binding cassette (ABC) transporter capable of causing cancer drug resistance. In one such cell line (MCF-7/AdrVp), the over expression of a novel member of the G subfamily of ABC transporters was found. The new transporter was termed the breast cancer resistance protein (BCRP), because of its identification in MCF-7 human breast carcinoma cells (Doyle and Ross 1998).

It was demonstrated that the presence of SP cells in the human adreno cortical tumor cell line NCIh295R and revealed an expression pattern consistent with a less differentiated phenotype, including lower steroidogenic enzyme such as steroid acute regulatory protein (StAR) and side chain cleavage enzyme (P450SCC) in comparison with non-SP cells. In addition, they found out that proliferation between SP and non-SP did not differ. (Urs *et al.*, 2008)

It was investigated whether SP cells reside within embryonic tissues and exhibit hematopoietic progenitor activity. It was suggested that, yolk sac and embryonic SP cells before the onset of circulation express the highest levels of endothelial markers and as the development progresses, they acquire hematopoietic potential and phenotypic characteristics similar to those of bone marrow SP cells. (Nadin *et al.*, 2003)

Considerable attention was drawn to the role played by membrane transporter proteins belonging to the ATP binding cassette protein super family, and in particular by

the MDR1 product p-glycoprotein (Pgp) and the multidrug resistance protein (MRP1). Exposure to chemotherapy increases the expression of both proteins. In vitro studies on primary cultures of breast cancer cells obtained at surgery consistently show an association between Pgp (protein) or MDR1 (mRNA) expression and resistance to chemotherapy (Leonessa and Clarke 2003)

It was discovered that the human ATP-binding cassette super family G (White) member 2 (*ABCG2*) gene and its murine homologue breast cancer resistance protein 1 (BCRP1) are recently described ATP-binding cassette transporters associated with drug resistance in tumor cell lines, including the MCF-7 cell line. It was also identified that a primitive hematopoietic stem cell population, or side population (SP) cells, which are identified by their efflux of the fluorescent dye, Hoechst 33342 (Kenneth *et al.*, 1996).

Efforts were carried out to improve severely impaired myocardial function include transplantation of autologous hematopoietic side population (SP) stem cells. The transmembrane ABC-type (ATP binding cassette) half-transporter ABCG2 (BCRP) serves as a marker protein for SP cell selection. We have recently shown that other ABC transport proteins such as ABCB1 and ABCC5 are differentially expressed. (Konrad *et al.*, 2006)

It was found that SP cells can be isolated by dual-wavelength flow cytometry because of their capacity to efflux Hoechst dye, a process mediated by the ATP-binding cassette transporter breast cancer resistance protein (BCRP). (Ross *et al.*, 2003). The two ABC-transporter-encoding genes that have been studied most extensively in stem cells are ABCB1, which encodes p-glycoprotein, and ABCG2. Along with ABCC1, they represent the three principal multidrug-resistance genes that have been identified in tumor cells. These genes, members of the ABC-transporter super family, are promiscuous transporters of both hydrophobic and hydrophilic compounds. The term side population was coined because during flow-cytometry analysis, SP cells are visualized as a negatively stained 'side population' to one side of the majority of cells on a density dot plot. SP cells can be isolated from many tissues including the brain, breast, lung, heart,

pancreas, testes, skin and liver, and these cells might represent lineage-specific stem cells.

It was standardized that this method is based on the over expression of transmembrane transporters, like the ATP-binding cassette molecules ABCG2/BCRP1. In stem cells, these molecules exclude vital dyes such as Hoechst 33342 or rhodamine 123 from the cells, a property not found in differentiated cells that remain positive for the dye. A side population has been isolated from the breast cancer cell line MCF7, representing 2% of the total cell line and containing the only tumorigenic fraction from MCF7 (Daniel *et al.*, 2008)

Experiments on cancer cells showed that 30% of cultured human cancer cells and xenograft tumors possess a detectable side population and the purified side population cells from two cell lines (U373 glioma and MCF7 breast cancer) and a xenograft prostrate tumor (LAPC-9) are found to be more tumorigenic than the corresponding non-side population cells. Their experiments suggested that the side population is enriched with tumorigenic stem-like cancer cells and they showed that in contrast to the tumorigenic differences between the side population and non-side population cells, the ABCG2+ and ABCG2- cancer cells show very similar tumorigenicities in vivo. (Lubna *et al.*, 2005)

It was demonstrated that placental P-gp (protein and mRNA) expression is highest early in gestation (60–90 days) and falls dramatically at term. Thus, early in pregnancy, P-gp appears to play an important role in modulating exposure of the fetus to xenobiotics that are transported by P-gp. n increase in transcripts of the nuclear receptor factors such as PXR, CAR, HNF-4β, and VDR is not the mechanism by which placental P-gp expression is increased in early pregnancy. Interestingly, expression of human placental hCG-β demonstrates a temporal pattern similar to that of expression of P-gp. Therefore, further studies need to be conducted to determine the role of hCG-β in the regulation of placental P-gp. (Anita *et al.*, 2005)

The human ATP-binding cassette superfamily G (White) member 2 (ABCG2) gene and its murine homologue breast cancer resistance protein 1 (BCRP1) are recently described ATP-binding cassette transporters associated with drug resistance in tumor cell lines, including the MCF-7 cell line, selected for its resistance to mitoxantrone (MCF-7/MitoR). Infection of MCF-7 cells with the retroviral vector containing ABCG2 cDNA (G1-ABCG2) resulted in cells (MCF-7/ABCG2) that were resistant to mitoxantrone at levels similar to those observed in MCF-7/MitoR cells. The level of the mouse BCRP1 RNA in SP cells and non-SP cells isolated from mouse hematopoietic cells were examined. Mouse SP cells expressed relatively high levels of BCRP1 mRNA relative to non-SP cells was also examined. These results suggest that Hoechst 33342 is a substrate for the ABCG2 transporter and that ABCG2/Bcrp1 expression may serve as a marker for hematopoietic stem cells in hematopoietic cells. (Min *et al.*, 2002)

It was studied that SP from bone marrow, as well as other tissues, is reported to contain immature stem cells with considerable plasticity. Some cell lines also efflux Hoechst and generate SP profiles. Reverse transcription– polymerase chain reaction (RT-PCR) and efflux inhibition studies with the lung carcinoma cell line, A549, implicated the ABCG2 transporter as a Hoechst efflux pump. Furthermore, it is shown that transient expression of ABCG2 generates a robust SP phenotype in human embryonic kidney (HEK293) cells. The results allow the conclusion that ABCG2 is a potent Hoechst efflux pump. (Robert 2005)

Comparison of messenger RNA (mRNA) levels for the 3 major multidrug-resistant efflux pumps, MDR1, MRP1, and ABCG2, in bone marrow SP cells reveals that ABCG2 is the predominant form in these cells. These data suggest thatABCG2 contributes significantly to the generation of the SP phenotype in hematopoietic stem cells. Furthermore, the sharp down-regulation of ABCG2 at the stage of lineage commitment suggests that this gene may play an important role in the unique physiology of the pluripotent stem cell. (Christian *et al.*, 2002)

When cell populations are incubated with the DNA-binding dye Hoechst 33342 and subjected to flow cytometry analysis for Hoechst 33342 emissions, active efflux of the dye by the ABCG2/BCRP1 transporter causes certain cells to appear as a segregated group. Sorted cells were subjected to several tests to determine whether the isolated SP cells displayed features consistent with the stem cell phenotype. Side populations amounting to <1% of total cells, which were sensitive to the ABCG2-inhibitor fumitremorgin C, were found in the conjunctival and limbal epithelia, but were absent from the stem cell-free corneal epithelium. Immunohistochemistry was used to establish the spatial expression pattern of ABCG2. SP cells were characterized by extremely low light side scattering and contained a high percentage of cells that: showed slow cycling prior to tissue collection; exhibited an initial delay in proliferation after culturing; and displayed clonogenic capacity and resistance to phorbol-induced differentiation; all features that are consistent with a stem cell phenotype. (Murat *et al.*, 2005)

Multidrug resistance in Chinese hamster ovary cells is associated with the M, 170,000 surface glycoprotein. Southern blot analysis shows that the p-glycoprotein sequences are greatly amplified in resistant Chinese hamster ovary cells but not in the resistant human breast cancer cells, indicating that amplification and expression of the M, 170,000 p-glycoprotein genes are not necessarily coordinate events. Amplification of this gene may not be required for multidrug resistance in human cells. Though the degree of cross-resistance and the drugs involved in the process of MDR vary among different cell lines, suggesting a multicomponent, complex system, there is considerable evidence to link the overproduction of the p-glycoprotein to MDR. (Suzanne *et al.*, 1987)

Relapse is common in acute myeloid leukemia (AML) because of persistence of minimal residual drug (MRD). ABC transporters p-glycoprotein and MRP (multidrug resistant protein) are thought to contribute to treatment failure. Emergence of MRD is not accompanied by upregulation of ABC transporters function during chemotherapy. The prognostic value of P-gp and MRP is likely related to drug efflux capacity. BCRP1 has limited fuction in drug efflux rated resistance in AML. (Bijan *et al.*, 2003)

Human sarcoma lines were examined for their sensitivity to common antitumor drugs and expression of putative multidrug resistance (MDR) proteins. A relationship between MDR1 mRNA expression and response to doxorubicin was demonstrated in >90% of our tumor lines. In six sarcomas with MDR1 mRNA expression, five were resistant against doxorubicin and cross-resistant against several other drugs, whereas from eight sarcomas, which lacked detectable mdr1 mRNA, seven were sensitive to doxorubicin and other drugs. We found lung resistance protein or MDR associated protein expressed in three resistant and mdr1 mRNA-positive sarcomas. These results demonstrate that MDR1 mRNA expression is a putative marker for drug resistance in our sarcoma lines and that inherent P-glycoprotein expression might be also responsible for drug resistance occurring in treatment of patients with sarcomas. (Jens *et al.*, 1999)

Studies were carried out on 42 different sublines for some basic characteristics namely cross resistance in other anti cancer drug investigated by in vivo MTT assay, expression of MDR associated protein (P-gp, MRP1, LRP) as well as functional activity of P-gp and MRP. (Noskova *et al.*, 2002)

It was studied that CD44+CD24- stem cell phenotype is associated with basal-type breast cancers in human patients; in particular *BRCA1* inherited cancers, but does not correlate with clinical outcome. Given the large representation of CD44+CD24- cells in the basal layer of normal breast epithelium, these findings suggest that the cellular origin of the basal breast cancers may be a progenitor of the basal lineage. This is also consistent with the recently proposed role of BRCA1 in luminal differentiation. Alternatively, or in addition, a block in luminal differentiation may lead to the same basal-like molecular phenotype, which often contains CD44+CD24- cells. However, not all basal breast tumors and very few HER2 positive tumors had CD44+CD24- cells. (Gabriela 2008)

MATERIALS AND METHOD

4. MATERIALS AND METHODS

4.1 MATERIALS

1. Cell line- MCF7

   Isolated in 1970 from a 69year old Caucasian woman.

Tumor type	: Infiltrating Ductal Adenocarcinoma
Tissue source	: Pleural effusion
Cell type classification	: Luminal
Properties	: ER+ and PR+

2. Hoechst33342 (Gibco)
3. Verapamil (Sigma)
4. Dulbecco's Minimum Essential Medium (DMEM) (Sigma)
5. HBSS (Sigma)
6. 0.25% Trypsin (Sigma)
7. Propidium Iodide (Sigma)
8. FACS tubes (BD Falcon)
9. 40 µcell strainer (BD Falcon)
10. TritonX-100
11. T25 flasks (Greiner)
12. Goat ant mouse IgG- PE (Santacruz)
13. Fetal Bovine Serum (Gibco)

4.2 MAINTENANCE OF CELL LINE MCF7

Human breast cancer cell line MCF-7 was maintained in DMEM (Dulbecco's Modified Eagle's Medium; Sigma Aldrich) supplemented with 1% penicillin/streptomycin and 10% Fetal Bovine Serum (FBS, Gibco). Cells were maintained in humidified incubator at 37°C supplied with 5% carbon dioxide. Cells were routinely maintained in 75 cm^2 and 25 cm^2 tissue culture flasks (Greiner) and harvested using 0.25% trypsin (Sigma Aldrich) treatment for side population analysis (Fig 4.1).

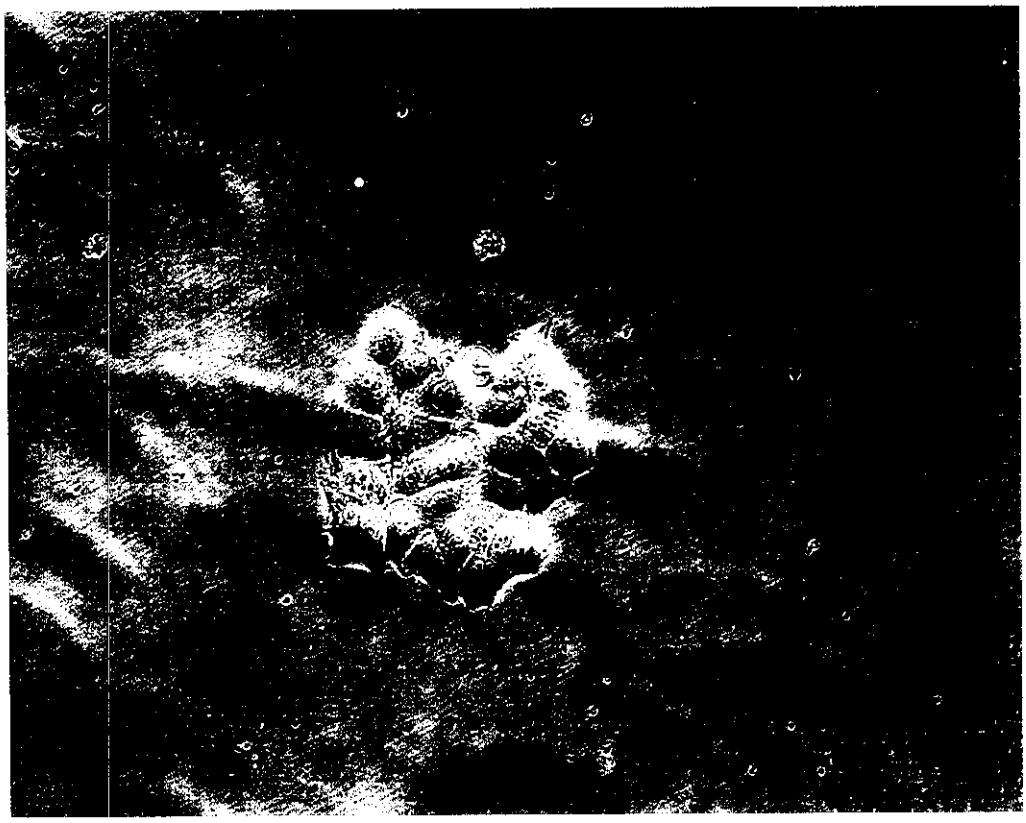


Fig 4.1 MCF7 Cells

4.3 FLOW CYTOMETRY

Flow cytometry uses the principle of light scattering, light excitation and emission of fluorochrome molecules to generate specific multiparameter data from particles and cells in the size range of 0.5m to 40m diameter. Cells are hydro dynamically focused in a sheath of PBS before intercepting an optimally focused light source. Lasers are most often used as a light source.

As the cells or particles of interest intercept the light source, they scatter light and fluorochrome is excited to a higher state. Flow cytometry measures fluorescence per cell or particle. Scattered and emitted light from cells and particles are converted to electrical pulses by optical detectors. Light is send to different detectors by using optical filters.

4.4 SIDE POPULATION ANALYSIS AND SORTING USING FLOW CYTOMETRY

Samples:

Control : Cells + Hoechst33342

Drug treated : Cells + Hoechst33342+Verapamil

1. Cells on becoming 80-85% confluent were removed from the T-25 flask using 0.25% Typsin EDTA.
2. Trypsined cells were re suspended at 10^6cells/ml in DMEM supplemented with 2%FBS pre warmed to 37°C.
3. Hoechst33342 (Gibco) was added at a final concentration of 5µg/ml. In the drug treated set Verapamil (Sigma) was added at a final concentration of 75µM to inhibit Hoechst efflux.
4. Cells were incubated at 37°C with intermittent mixing.
5. After incubating cells were pelleted down and resuspended in cold HBSS supplemented with 2% FBS.
6. Cells were then sieved through a 40 µcell strainer (BD Falcon) into labeled FACS tubes (BD Falcon) and stained with 2µg/ml Propidium Iodide (Sigma).
7. Final analysis and sorting was done in BDFACS Aria sorter (Becton Dickinson).

8. Hoechst33342 fluorescence was measured at 424nm and 670nm both from UV excitation.
9. Dead cells were excluded by Propidium Iodide fluorescence measured at 564nm and 605nm.

4.5 IMMUNOFLUORESCENCE ANALYSIS OF SIDE POPULATION

1. Single cell suspension of SP cells and Non SP cells were seeded onto petri dishes and allowed to grow using the same conditions already described.
2. After 7 days, the cells were removed from T-25 flask using 0.25% Trypsin EDTA.
3. Trypsinized cells were resuspended in 1ml of 10%DMEM.
4. This suspension is centrifuged at 3000rpm for 7min at 27°C.
5. The pellet is suspended in 1ml PBS.
6. Cells are incubated in an eppendorf for 15min at room temperature in 4% PFA-PBS.
7. This suspension is centrifuged at 3000rpm for 7min at 27°C.
8. Cells were incubated for 5mins at room temperature in 0.2% TritonX-100.
9. This suspension is centrifuged at 3000rpm for 7min at 27°C.
10. 3%BSA TBST is added to this and incubated at RT for 10mins.
11. This is centrifuged at 3000rpm for 8min at 27°C.
12. The pellet was incubated in primary antibody (1:1000 dilution) 1% BSA TBST at 37°C for 180min
13. This is then centrifuged at 3000rpm for 8min at 27°C.
14. The pellet was incubated in secondary antibody (1:200 dilution) 1% BSA TBST at 37°C for 60min.
15. This is finally centrifuged at 3000rpm for 8min at 27°C.
16. Resuspend the pellet using 1ml PBS and filtered through 40μ cell strainer.
17. Final analysis was done in BDFACS Aria sorter (Becton Dickinson).
18. PE fluorescence was measured at 564nm and 605nm.

RESULT AND DISCUSSION

5. RESULT AND DISCUSSION

5.1 ISOLATION AND SORTING OF SIDE POPULATION CELLS

Side population cells are a rare subset of cells with the unique ability to efflux lipophilic dye such as Hoechst 33342.

SP analysis and sorting was carried out using a dual wavelength cell sorter (BD Falcon Aria) based on Hoechst fluorescence. This dye is excited by UV laser at 350nm-395nm and its fluorescence was measured with both 450/50nm and 675/50nm filter.

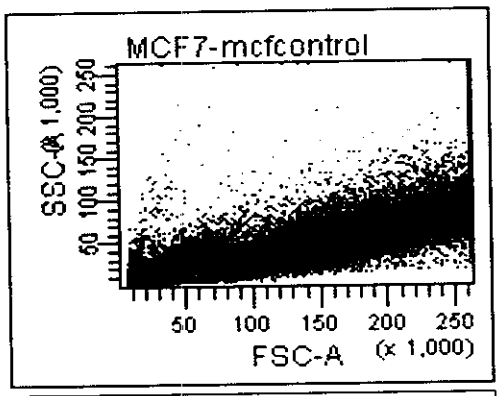
A live gate on the FSC-A and PI-A axes of the flow cytometry helped to exclude the dead population of cells.

A low Hoechst excluding population was identified by adjusting the SP-Violet and SP-Red axes. The analysis showed that 1.1% of low Hoechst was present in the control and this was confirmed by the analysis of Verapamil treated cells (Fig 5.1 & 5.2).

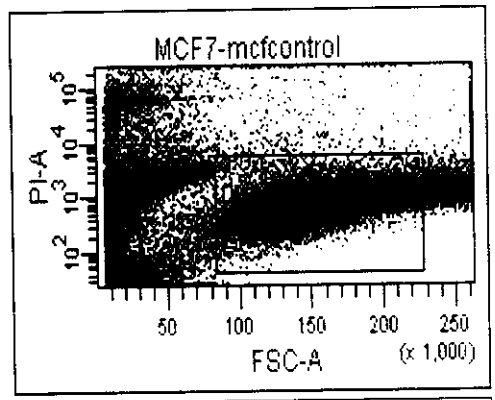
The dye exclusion property of SP cells is partly due to the functional ABC transporters and verapamil effectively blocking the ABC membrane transporter from extruding the Hoechst dye (Min et al., 2002).

Sorted SP cells exhibited small spindle-like features and formed colonies when cultured in the condition with supplementations, on the other hand, Non SP cells did not form colonies, and the proliferation speed was low (Takeshi *et al.*, 2008) (Fig 5.3 & 5.4).

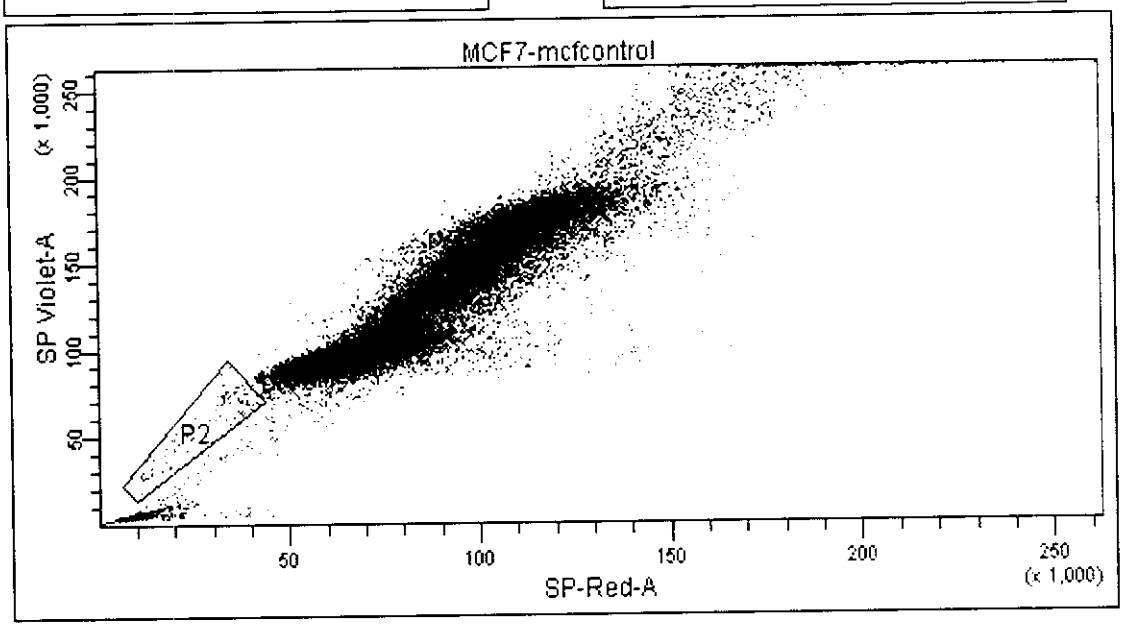
The following graphs were obtained from the FACS machine while sorting side population cells.



The scatter of the cells is distinguished based on their size.



The P1 gate shows the live and viable population of cells



The P2 gate shows the side population cells.

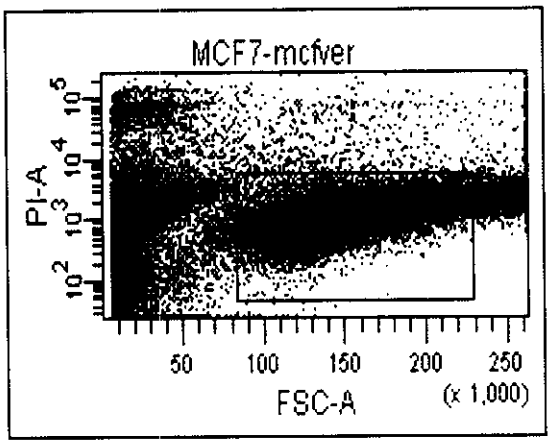
Tube: mcfcontrol

Population	#Events	%Parent	%Total
All Events	103,100		100.0
P1	48,369	46.9	46.9
P2	548	1.1	0.5

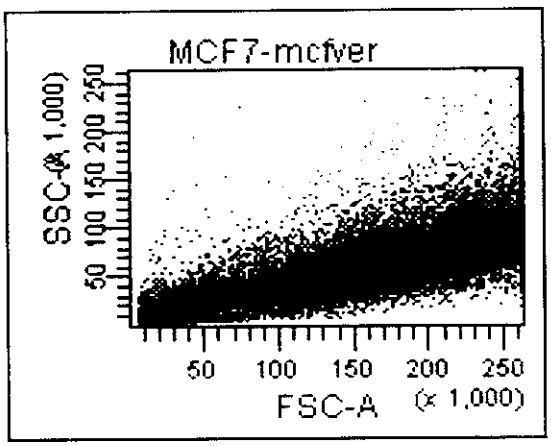
1.1% cells were isolated as side population cells

Fig 5.1 MCF7 Control

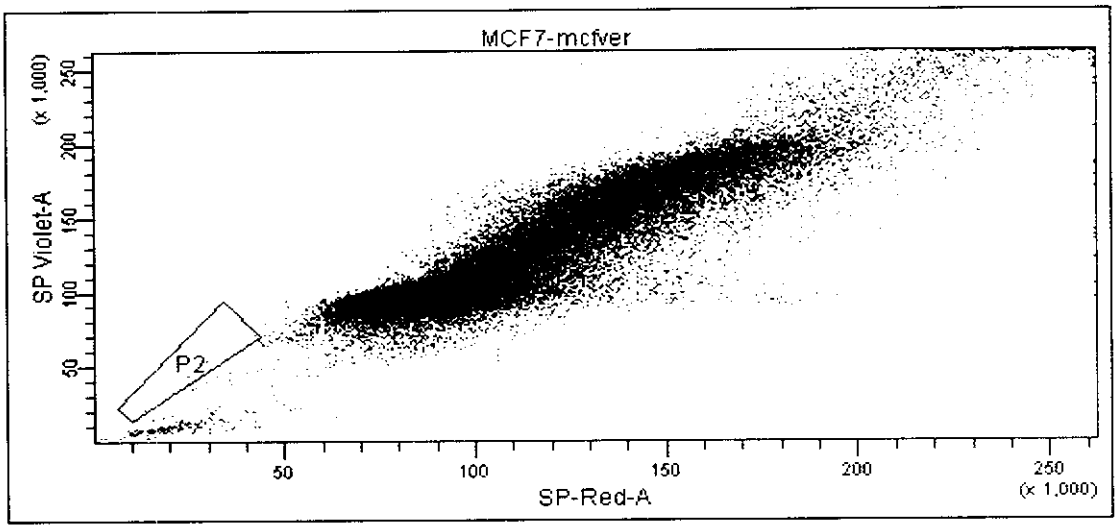
The following graph shows cells that have been treated with Verapamil



The P1 gate shows the live and viable population of cells.



The scatter of the cells is distinguished based on their size.



The P2 gate shows that 1% of the cells have been shifted after treatment with Verapamil.

Tube: mcfver

Population	#Events	%Parent	%Total
All Events	93,934		100.0
P1	49,076	52.2	52.2
P2	32	0.1	0.0

1% of the excluded from the side population

Fig 5.2 MCF7 Verapamil Treated

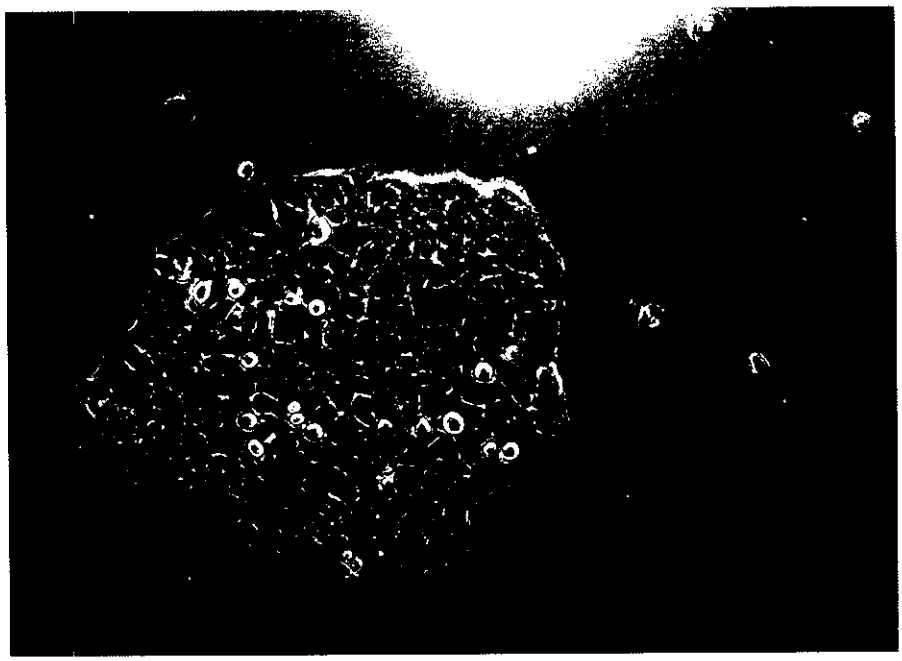


Fig 5.3 Growth of side population cells after 48hrs

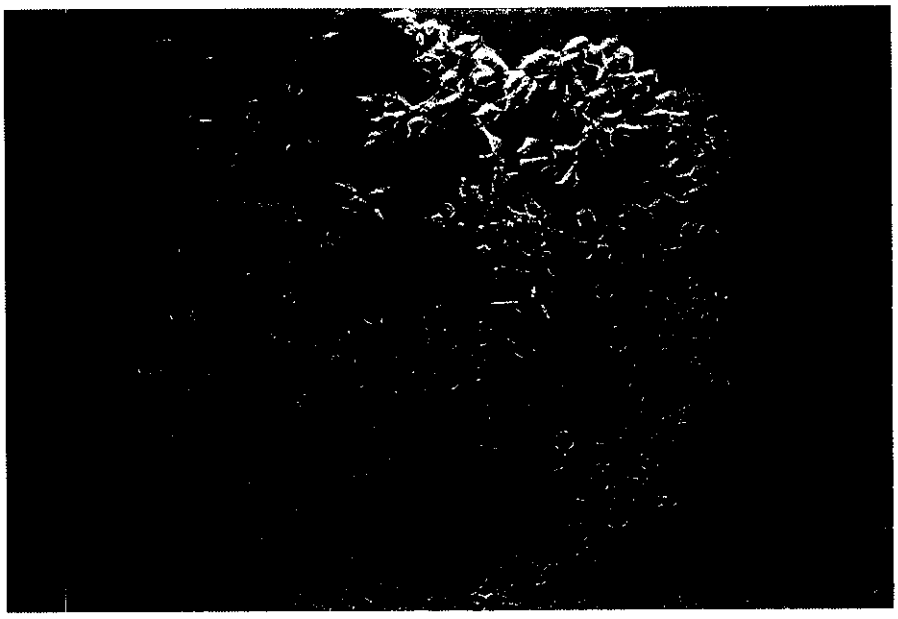


Fig 5.4 Growth of non-side population cells after 48hrs

5.2 IMMUNOPHENOTYPING

Immunophenotypic analysis of the sorted SP and Non SP fractions was done using the membrane marker.

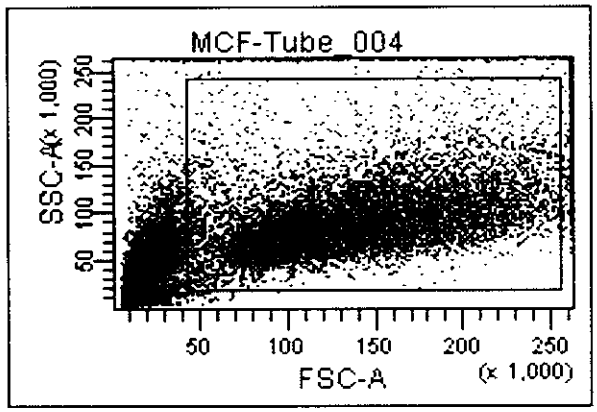
Characterization of sorted cells helps us to know, the proteins that are enriched in side population.

From the analysis it was found that MCF-7 contains side population cells and the SP fraction is enriched for certain MDR (Multi Drug Resistant) proteins.

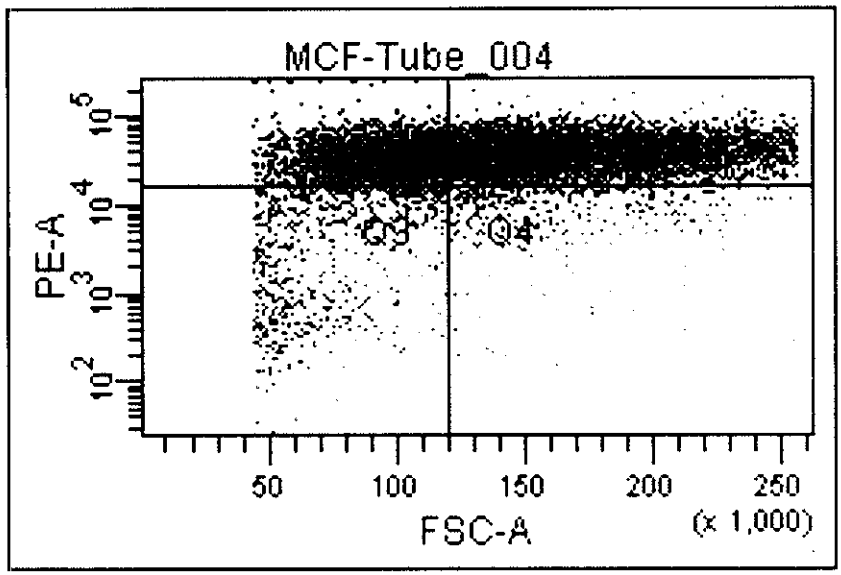
ABCG2 and MDR1 transmembrane markers showed a good expression in Side Population cells. ATP binding cassette transporter, ABCG2, has been shown to contribute to produce an SP phenotype in hematopoietic stem cells (Zhou *et al.*, 2001). It has been suggested that the SP phenotype may be caused by expression of the MDR1 gene (Goodell *et al.*, 1996) (Fig 5.5, 5.6, 5.7 & 5.8).

MRP showed very little difference in expression between the Side Population cells and Non Side Population cells. It is almost certainly unrelated to the presence of many of the ATP-binding cassette (ABC) transporter proteins such as MRP, have normal numbers of SP cells (Uchida *et al.*, 2002) (Fig 5.9 & 5.10).

The following graph shows the expression of ABCG2 maker in Side Population cells.



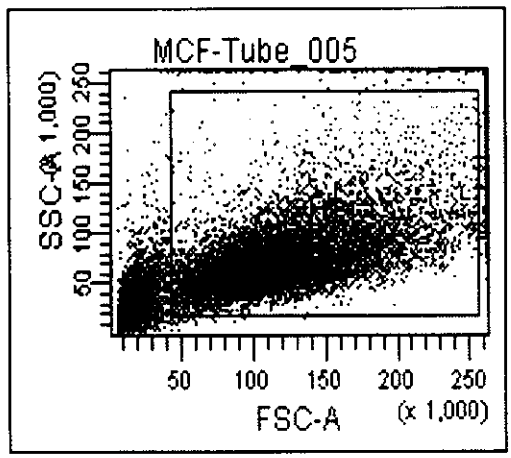
The cells are distinguished based on their size.



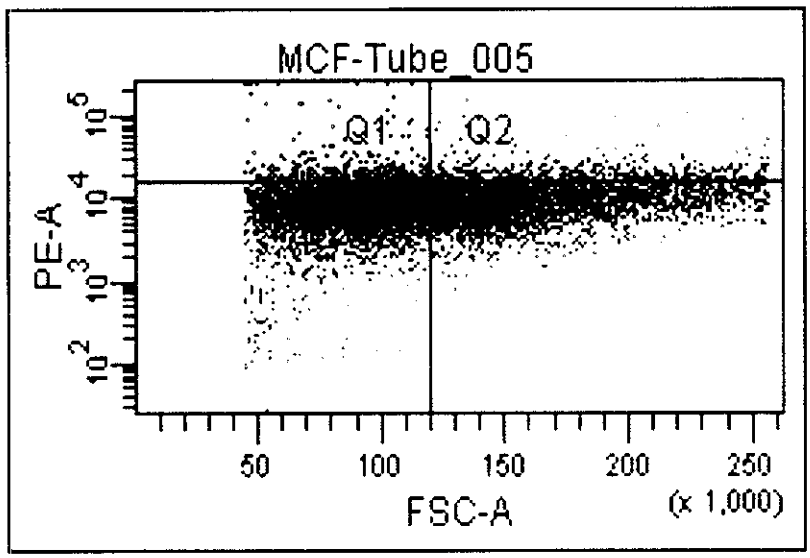
Q1 quadrant shows 28% of ABCG2 expression

Fig 5.5 ABCG2 SP

The following graph shows the expression of ABCG2 maker in Non Side Population cells.



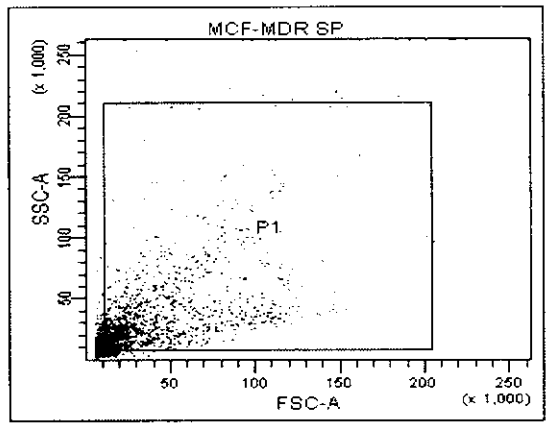
The cells are distinguished based on their size.



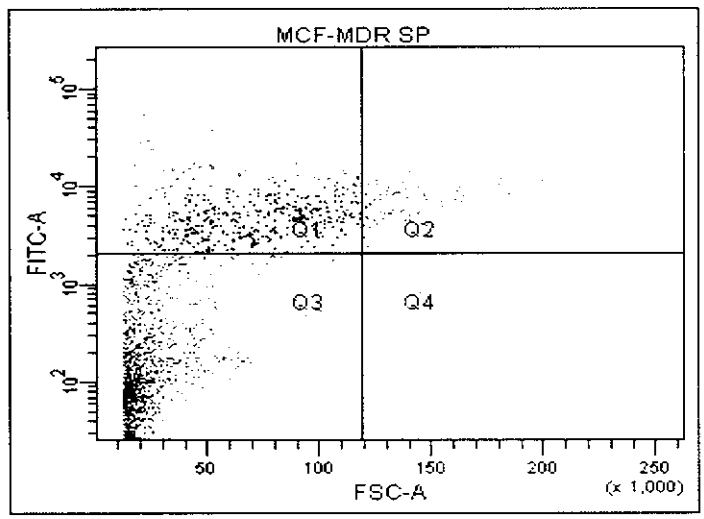
Q1 quadrant shows 7.2% of ABCG2 expression

Fig 5.6 ABCG2 Non SP

The following graph shows the expression of MDR1 maker in Side Population cells.



The cells are distinguished based on their size.



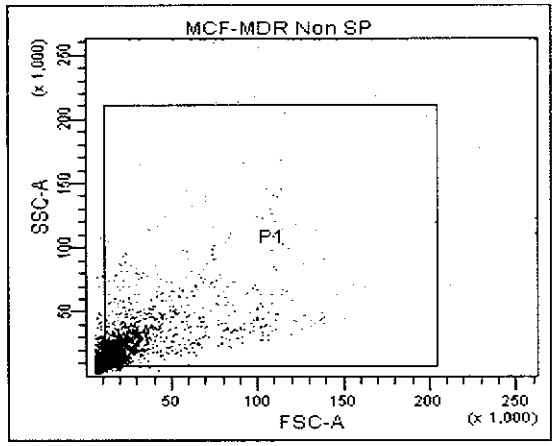
The Q1 quadrant shows 26.8% of MDR expression

Tube: MDR SP

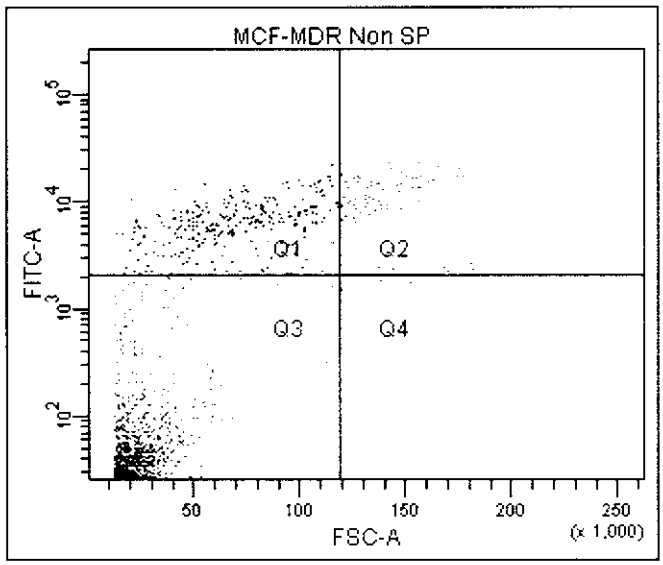
Population	#Events	%Parent	%Total
All Events	7,323		100.0
P1	1,764	24.1	24.1
Q1	472	26.8	6.4
Q2	98	5.6	1.3
Q3	1,190	67.5	16.3
Q4	4	0.2	0.1

Fig 5.7 MDR1 (P-GLYCOPROTEIN) SP

The following graph shows the expression of MDR1 maker in Side Population cells.



The cells are distinguished based on their size.



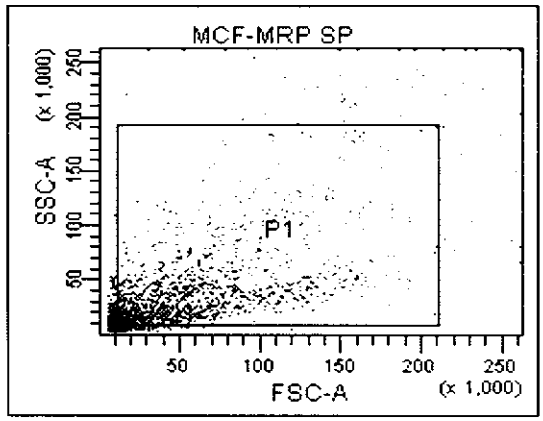
The Q1 quadrant shows 13.1% of MDR1 expression

Tube: MDR Non SP

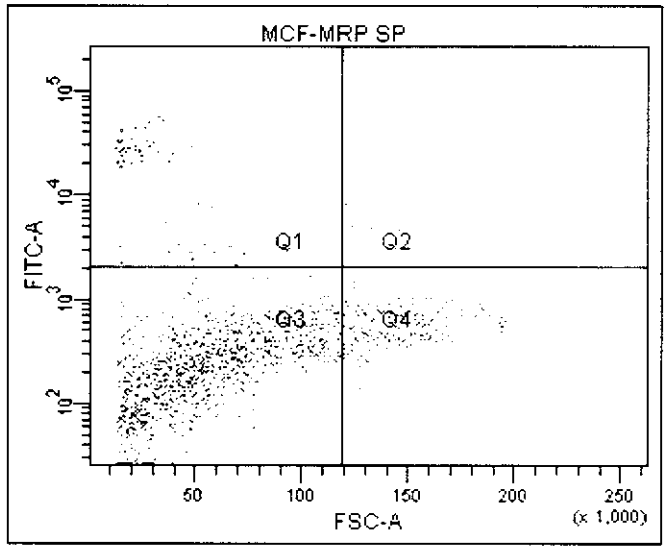
Population	#Events	%Parent	%Total
All Events	8,217		100.0
P1	2,142	26.1	26.1
Q1	280	13.1	3.4
Q2	91	4.2	1.1
Q3	1,765	82.4	21.5
Q4	6	0.3	0.1

Fig 5.8 MDR1 (P-GLYCOPROTEIN) NON SP

The following graph shows the expression of MRP maker in Side Population cells.



The cells are distinguished based on their size.



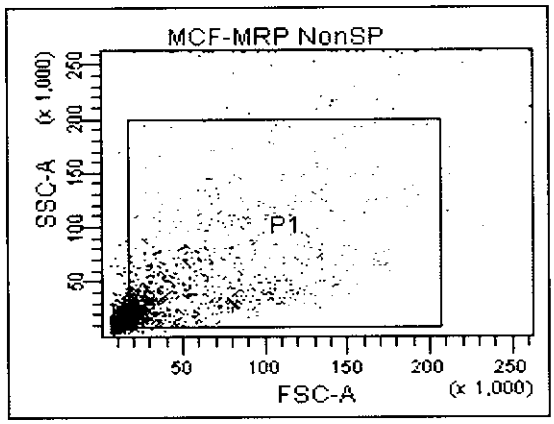
The Q1 quadrant shows 4.7% of MRP1 expression

Tube: MRP SP

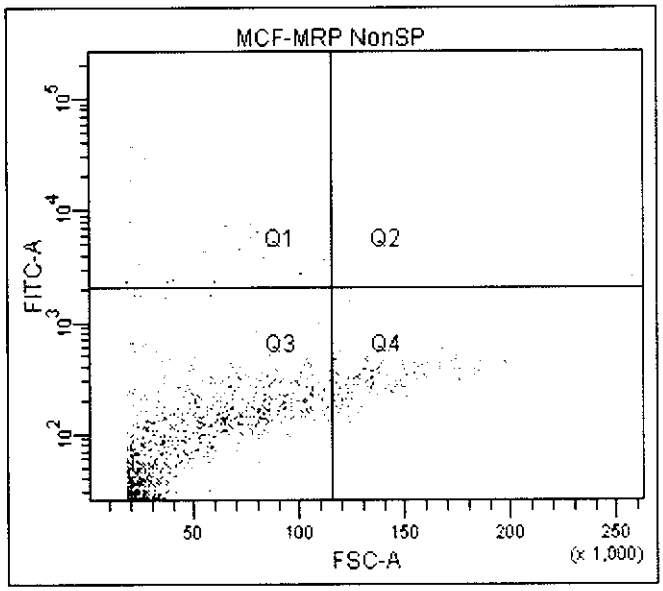
Population	#Events	%Parent	%Total
All Events	1,928		100.0
P1	1,225	63.5	63.5
Q1	57	4.7	3.0
Q2	11	0.9	0.6
Q3	976	79.7	50.6
Q4	181	14.8	9.4

Fig 5.9 MRP SP

The following graph shows the expression of MRP maker in Non Side Population cells.



The cells are distinguished based on their size.



The Q1 quadrant shows 1.9% of MRP expression

Tube: MRP NonSP

Population	#Events	%Parent	%Total
All Events	6,897		100.0
P1	1,132	16.4	16.4
Q1	22	1.9	0.3
Q2	2	0.2	0.0
Q3	963	85.1	14.0
Q4	145	12.8	2.1

Fig 5.10 MRP NON SP

CONCLUSION

6. CONCLUSION

Human breast cancer cell line MCF7 was cultured and analyzed for side population cells using BD FACS Aria cell sorter. The analysis showed that in $1x10^6$ population of MCF7 cells, 1.1% of side population cells were present. Sorted cells were cultured for 7 days and their phenotypic characterization showed that they were enriched for transmembrane markers like ABCG2, MRP and MDR1.

APPENDICES

APPENDICES

1. DULBECCO'S MODIFIED EAGLES MEDIUM (Sigma Aldrich) : 1000ml

DMEM (without $NaHCO_3$)	:	3.0g
$NaHCO_3$	:	3.6g
Autoclaved distilled water	:	1000ml

Media is filtered through a 0.2 µm membrane filter and aliquot into sterile 50mL screw cap tubes. Stored at 4°C

2. 10% DMEM (Sigma Aldrich) : 1000ml

Heat inactivated FBS	:	100ml
Plain DMEM	:	900ml

3. PBD-EDTA : 1000ml

NaCl	:	8.0g
Na_2HPO_4	:	1.41g
KH_2PO_4	:	0.2g
KCl	:	0.2g
EDTA	:	0.197g
Autoclaved distilled water	:	1000ml

4. 0.25% Trypsin-EDTA (Sigma Aldrich) : 1000ml

NaCl	:	8.0g
Na_2HPO_4	:	1.41g
KH_2PO_4	:	0.2g
KCl	:	0.2g
EDTA	:	0.197g
Trypsin	:	2.5g
Autoclaved distilled water	:	1000ml

5. 4% PARAFORMALDEHYDE :1000ml

NaCl	:	8.0g
Na_2HPO_4	:	1.41g
KH_2PO_4	:	0.2g
KCl	:	0.2g
Paraformaldehyde	:	40g
Autoclaved distilled water	:	1000ml

Warm the solution at 70°C till it turns clear liquid.

6. 10X TBS : 1000ml

Tris base	:	60.57g
NaCl	:	61.362g
Autoclaved distilled water	:	1000ml

7. 3% BSA TBST : 1000ml

Tris base	:	6.057g
NaCl	:	6.136g
Tween 20	:	5.0ml
BSA	:	3.0g
Autoclaved distilled water	:	1000ml

8. 1% BSA TBST

Tris base	:	6.057g
NaCl	:	6.136g
Tween 20	:	5.0ml
BSA	:	10.0g
Autoclaved distilled water	:	1000ml

REFERENCES

REFERENCE

1. Al-Hajj M., M. S. Wicha, A. Benito, S. J. Morrison and M. F. Clarke (2003) 'Prospective identification of tumorigenic breast cancer cells' Proceedings of National Academy of Science USA Vol.100, No.7, pp. 3983–3988.
2. Anita A. M., J. Hitti and J. D. Unadkat (2005) 'P-glycoprotein and breast cancer resistance protein expression in human placentae of various gestational ages' Am J Physiol Regul Integr Comp Physiol Vol.289, pp.R963-R969
3. Austin D. and D. D. Ross (2003) 'Multidrug resistance mediated by the breast cancer resistance protein BCRP (ABCG2)' Oncogene Vol.22, pp.7340–7358.
4. Azra J. A., H. Clayton, C. Joshi, T. Enver, A. Ashworth, M. M. Vivanco, T. C. Dale, and M. J. Smalley (2003) 'Functional and molecular characterisation of mammary side population cells' Breast Cancer Res Vol.5, No.1, pp. R1–R8.
5. Bijan M., A. V. Rhenen, A. Kelder, M. V. Pol, M. Terwijn, C. Bachas, A. H. Westra, G. J. Ossenkoppele, S. Zweegman and G. J. Schuurhuis (2008) 'Identification of a Small Subpopulation of Candidate Leukemia-Initiating Cells in the Side Population of Patients with Acute Myeloid Leukemia' Cancer Stem cells Vol.26, No.12, pp.567-578.
6. Bunting K. D., S. Zhou, J. Lu and B. P. Sorrentino (2000) 'Enforced p-glycoprotein pump function in murine bone marrow cells results in expansion of side population stem cells *in vitro* and repopulating cells *in vivo*' Blood Vol.96, pp.902-909.
7. Cheol- H. C. (2005) 'ABC transporters as multidrug resistance mechanisms and the development of chemosensitizers for their reversal' Cancer cell Vol.5, pp.68-72.
8. Christian W. S., M. A. Harkey and B. T. Storb (2002) 'The ABCG2 transporter is an efficient Hoechst 33342 efflux pump and is preferentially expressed by immature human hematopoietic progenitors' Blood Vol.99, pp.507-512.

9. Christine M. F. and C. Kuperwasser (2008) 'Human Breast Cancer Cell Lines Contain Stem-like Cells That Self-renew, Give Rise to Phenotypically Diverse Progeny and Survive Chemotherapy, Medscape today Vol.109, pp.389-395.
10. Coppock H. A. and R. B. Clarke (2004) 'Mammary stem cells: the root of breast cancer?' Breast Cancer online Vol.14.
11. Daniel B., E. C. Jauffret, F. Monville, C. Ginestier, G. Dontu and M. S. Wicha (2008) 'Cancer Stem Cells in Breast: Current Opinion and Future Challenges' Pathobiology Vol.75, pp.75–84.
12. Dean M., Y. Hamon and G. Chimini (2001) 'The human ATP- binding cassette (ABC) transporter super family' J Lipid Res Vol.42, pp.1007-1017.
13. Deely R. G. and S. P. Cole (1997) 'Function, evolution and structure of multi drug resistance protein (MRP)' Seminars in Cancer Biology Vol.8, pp.193-204.
14. Doyle L. A. and D. D. Ross (2003) 'Multidrug resistance mediated by the breast cancer resistance protein BCRP (ABCG2)' Oncogene Vol.22, No.47, pp.7340–7358.
15. Fariba B., W. Xian, C. A. Shaw, S. G. Hilsenbeck, A. Tsimelzon and J. M. Rosen (2006) 'Transcriptional Profiling of Mammary Gland Side Population Cells' Stem Cells Vol. 24, No. 4, pp. 1065 -1074.
16. Gabriela D. (2008) 'Breast cancer stem cell markers - the rocky road to clinical applications' Breast Cancer Research Vol.10, pp.110-120.
17. Goodell M. A., K. Brose, G. Paradis, A. S. Conner, R. C. and Mulligan (1996) 'Isolation and functional properties of murine hematopoietic stem cells that are replicating *in vivo*' JEM Vol.183, pp.1797-1806.
18. Grant A. C. and M. H. Little (2006) 'A Side Order of Stem Cells: The SP Phenotype' Stem Cells Vol.24, No.1, pp.3-12.
19. Higgins C. F, R. Callaghan, K. J. Linton, M. F. Rosenberg, R. C. Ford (1997) 'Structure of multidrug resistance p-glycoprotein' Seminars in Cancer Biology Vol.8, pp.135- 142.
20. Iwatani H, T. Ito, E. Imai, Y. Matsuzaki, A. Suzuki, M. Yamato, M. Okabe and M. Hori (2004) 'Hematopoietic and nonhematopoietic potentials of

Hoechst(low)/side population cells isolated from adult rat kidney' Kidney Int Vol.65, pp.1604-1614.

21. Jens H., P. S. Peter, W. Hänsch, H. Naundorf, A. Bunge, M. Becker and I. Fichtner (1999) 'Anticancer Drug Sensitivity and Expression of Multidrug Resistance Markers in Early Passage Human Sarcomas' Clinical Cancer Research Vol. 5, pp.345-355.
22. Jiangbing Z., J. Wulfkuhle, H. Zhang, P. Gu, Y. Yang, J. Deng, J. B. Margolick, L. A. Liotta, E. Petricoin, and Y. Zhang (2007) 'Activation of the PTEN/mTOR/STAT3 pathway in breast cancer stem-like cells is required for viability and maintenance' Proceedings of National Academy of Science U S A. Vol.104, No.41, pp.16158–16163.
23. Jihyun Y., S. C. Choi, C. Y. Park, J. H. Choi, Y. I. Kim, W. J. Shim, and D. S Lim (2007) 'Bone Marrow-derived Side Population Cells are Capable of Functional Cardiomyogenic Differentiation' Mol. Cells Vol.25, No.2, pp. 216-223.
24. Jonathan B. J. and F. D. Camargo (2007) 'Isolation and Functional Characterization of Side Population Stem Cells' Methods in Molecular Biology Vol.430, pp.183-193
25. Karen S. A., J. Najbauer, N. O. Schmidt, W. Yang, J. K. Wu, Y. Zhuge, W. Przylecki, R. Carroll, P. M. Black and G. Perides (2005) 'Targeting of melanoma brain metastases using engineered neural stem/progenitor cells' Neuro Oncology Vol.10, pp. 1215-1220.
26. Katja E., H. Shen, and O. J. Finn (2008) 'MCF7 Side Population Cells with Characteristics of Cancer Stem/Progenitor Cells Express the Tumor Antigen MUC1' Cancer Research Vol.68, No.7, pp.2419–2426.
27. Kenneth C., H. Batist, G. Tulpule, A. Sinha and C. E. Myers (1996) 'Similar biochemical changes associated with multidrug resistance in human breast cancer cells and carcinogen-induced resistance to xenobiotics in rats' Proc. Nati. Acad. Sci. USA Vol.83, pp.9328-9332.
28. Kevin M. S., M. C. Winslet, and A. M. Seifalian (2007) 'Stem Cells and Cancer: An Overview' Stem cells review and reports Vol.3, No.4, pp.249-255.

29. Konrad M., B. Heydrich, G. Jedlitschky, H. Meyer, I. Mosyagin, P. Dazert, L. Eckel, S. Vogelgesang, R. W. Warzok, M. Bohm, C. Lehmann, M. Wendt, I. Cascorbi, and H. K. Kroemer (2006) 'The ATP-binding Cassette Transporter ABCG2 (BCRP), a Marker for Side Population Stem Cells, Is Expressed in Human Heart' Journal of Histochemistry and Cytochemistry Vol.54, No.2, pp. 215–221.

30. Leonessa F. and R. Clarke (2003) 'ATP binding cassette transporters and drug resistance in breast cancer' Endocrine-Related Cancer Vol.10, pp.43–73.

31. Lidia M., S. Indraccolo, M. Ghisi, C. Frasson, E. Fortunato, S. Canevari, S. Miotti, V. Tosello, R. Zamarchi, A. Corradin, S. Minuzzo, E. Rossi, G. Basso and A. Amadori (2008) 'The Side Population of Ovarian Cancer Cells Is a Primary Target of IFN-α Antitumor Effects' Cancer Research Vol.68, pp.5658-5668.

32. Liqun Y., P. Castagnino, and R. K. Assoian (2008) 'ABCG2 Expression and Side Population Abundance Regulated by a Transforming Growth Factor B–Directed Epithelial-Mesenchymal Transition' Cancer Res Vol.68, No.3, pp.800–807.

33. Louise H., F. Shaheen, A. Johnson, S. Wadsworth, V. D. Pechkovsky, D. B. Jacoby, A. Kicic, S. M. Stick and D. A. Knight (2008) 'Characterization of Side Population Cells from Human Airway Epithelium' Stem Cells Vol.26, No.10, pp.607-612.

34. Lubna P., T. Calhoun, R. S. Broussard, J. Zhou, K. Claypool, and D. G. Tang (2005) 'Side Population Is Enriched in Tumorigenic, Stem- Like Cancer Cells, whereas ABCG2+ and ABCG2_ Cancer Cells Are Similarly Tumorigenic' Cancer Research Vol.65, No.14, pp. 6207-6219.

35. Massard C., E. Deutsch and J. C. Soria (2006) 'Tumor stem cell-targeted treatment: elimination or differentiation' Annals of Oncology Vol.17, pp.1620–1624.

36. Matthew J. S. and R. B. Clark (2005) 'The Mammary Gland "Side Population": A Putative Stem/Progenitor Cell Marker?' Journal of Mammary Gland Biology and Neoplasia Vol.10, No.1, pp.37-47.

37. Meissner K., B. Heydrich, G. Jedlitschky, H. M. Schwabedissen, I. Mosyagin, P. Dazert, L. Eckel, S. Vogelgesang, W. R. Warzok, M. Bohm, C. Lehmann, M.

Wendt, I. Cascorbi and H. K. Kroemer (2006) 'The ATP binding cassette transporter ABCG2(BCRP), a marker for side population stem cells , is expressed in human heart' JHC Vol.54, pp.215-22.

38. Michael D., J. G. Sharp, and K. Cowan (2002) 'The Multidrug Resistance Transporter ABCG2 (Breast Cancer Resistance Protein 1) Effluxes Hoechst 33342 and Is Overexpressed in Hematopoietic Stem Cells' Clinical Cancer Research Vol.8, pp.22–28.
39. Min K., H. Turnquist, J. Jackson, M. Sgagias, Y. Yan, M. Gong, M. Dean, J. G. Sharp, and K. Cowan (2002) 'The Multidrug Resistance Transporter ABCG2 (Breast Cancer Resistance Protein 1) Effluxes Hoechst 33342 and Is Overexpressed in Hematopoietic Stem Cells' Clinical Cancer Research Vol.8, pp.22-28.
40. Molly A. H., H. Yang, B. E. Low, J. Mukherje, A. Guha, R. T. Bronson, L. D. Shultz, M. A. Israel and Y. Kyuson (2008) 'Cancer Stem Cells Are Enriched in the Side Population Cells in a Mouse Model of Glioma' Cancer Research Vol.68, No.24, pp.10051–10059.
41. Murat T. B., O. S. Alpdogan, M. Zhou, R. M. Lavker, M. A. Akinci and J. M. Wolosin (2005) 'Ocular surface epithelia contain ABCG2-dependent side population cells exhibiting features associated with stem cells' Journal of Cell Science Vol.118, pp.1715-1724.
42. Nadin B. M., M. A. Goodell and K. K. Hirschi (2003) 'Phenotype and hematopoietic potential of side population cells throughout embryonic development' Blood Vol.102, No.7, pp.2436-2443.
43. Naotsugu H., T. Utsunomiya, H. Inoue, F. Tanaka, K. Mimori, G. F. Barnard and M. Masaki (2006) 'Characterization of a Side Population of Cancer Cells from Human Gastrointestinal System' Stem Cells Vol.24, pp.506 –513.
44. Norisato M., A. Iwao, K. Nagai, H. Namba, A. Ohtsuru, V. Saenko and S. Yamashita (2007) 'Characterization of Side Population in Thyroid Cancer Cell Lines: Cancer Stem-Like Cells Are Enriched Partly But Not Exclusively' Endocrinology Vol. 148, No. 4, pp.1797-1803.

45. Noskova V., P. Dzubak, G. Kuzmina, A. Ludkova, D. Stehlik, R. Trojanec, A. Janostakova, G. Korinkova, V. Mihal and M. Hajduch (2002) 'In vitro chemoresistance profile and expression/function of MDR associated proteins in resistant cell lines derived from CCRF-CEM, K562, A549 and MDAMB 231 parental cells' Neoplasma Vol.49, No.6, pp.418-425.

46. Oyama T., T. Nagai , K. Matsuura, H. Wada, J. Nishi and I. Komuro (2005) 'Cardiac SP Cells Differentiate into Beating Cardiac Myocytes in vitro' Cir J Vol.69, No.1, pp.231-240.

47. Pfister O., A. Oikonomopoulos, K. I. Sereti, R. L. Sohn, D. Cullen, G. C. Fine, F. Mouquet, K. Westerman and R. Liao (2008) 'Role of the ATP-binding cassette transporter ABCG2 in the phenotype and function of cardiac side population cells' Circ. Res Vol.43, pp.76-88.

48. Robert. B. C. (2005) 'Isolation and characterization of human mammary stem cells' Cell Prolif Vol.38, pp.375–386.

49. Rosenberg M. F., Q. Mao, A. Holzenburg, R. C. Ford, R. G. Deeley and P. Susan, C. Cole (2001) 'The structure of the multidrug resistance protein 1(MRP1/ABCC1)' JBC Vol.276, pp.16076-16082.

50. Ross S., D. N. Kotton, X. Sun, B. Ma, K. Fitzsimmons, and A. Fine (2003) 'Side population cells and BCRP1 expression in lung' Am J Physiol Lung Cell Mol Physiol Vol.285, pp.L97–L104.

51. Sanjai K. A., M. D. Brown, C. A. Hart, V. A. Ramani and N. W. Clarke (2008) 'Characterization of the Hoechst 33342 side population from normal and malignant human renal epithelial cells' Am J Physiol Renal Physiol Vol.295, pp.F680-F687.

52. Sheng Z., J. D. Schuetz, K. D. Bunting1, A. M. Colapietro, J. Sampath, J. J. Morris, I. Lagutina, G. C. Grosveld, M. Osawa, H. Nakauchi4 and B. P. Sorrentino (2001) 'The ABC transporter BCRP1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype' Nature Medicine Vol.7, No.9, pp.1028-1034.

53. Shoichiro Y., Y. Ito, M. Fujimoto, T. S. Hamazaki, K. Tamaki and H. Okochi (2005) 'Characterization and Localization of Side Population Cells in Mouse Skin' Stem Cells Vol. 23,No.6, pp.834-841.

54. Suzanne A. W., I. M. Moretti, S. L. Schneides and W. L. M. Guire (1987) 'P-glycoprotein expression in human breast cancer cells' Cancer Research Vol.47, pp.2103-2106.

55. Takeshi T., K. Fukuda, S. Kurashimo, Y. Hoso, Y. Miki, S. Asada and C. Hamanishi (2008) 'Isolation and characterization of side population stem cells in articular synovial tissue' BMC Musculoskeletal Disorders Vol.9, No.86.

56. Terumasa U., M. Yamuto, K. Nishida, J. Yang, Y. Tano, T. Okano, M. Terasawa, Y. Shiratsuchi and Y. Kobayashi (2008) 'CD61 enriches long term repopulating hematopoietic stem cells' Biochemical and Biophysical research Vol.365, pp.176-180.

57. Toru K., T. Setoguchi, and T. Taga (2004) 'Persistence of a small subpopulation of cancer stem-like cells in the C6 glioma cell line' The National Academy of Sciences of the USA Vol.101, No.3, pp.781–786.

58. Uchida N., F. Y. Leung, C. J. Eaves (2002) 'Liver and marrow of adult mdr-1a/1b-/- mice show normal generation, function, and multi-tissue trafficking of primitive hematopoietic cells' Exp Hematol Vol.30, pp.862-869.

59. Urs D. L., I. Shapiro, K. Geiger, M. Quinkler, M. Fassnacht, R. Nitschke, K. D. Ruckauer, and F. Beuschlein (2008) 'Side Population Does Not Define Stem Cell-Like Cancer Cells in the Adrenocortical Carcinoma Cell Line NCI h295R' Endocrinology Vol.149, No.3, pp.1314–1322.

60. Wang J., L. P. Guo, L. Z. Chen, Y. X. Zeng and S. H. Lu (2007) 'Identification of Cancer Stem Cell–Like Side Population Cells in Human Nasopharyngeal Carcinoma Cell Line' Cancer Research Vol.67, pp.3716-3722.

61. Zhou S., J. D. Schuetz, K. D. Bunting, A. M. Colapietro, J. Sampath, J. J. Morris, I. Lagutina, G. C. Grosveld, M. Osawa, H. Nakauchi, and P. Sorrentino (2001) 'The ABC transporter BCRP1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side population phenotype' Nat Med Vol.7, pp.1028-1034.