CHARACTERIZATION OF CAL 27 ORAL SQUAMOUS CARCINOMA CELL LINE AS A MODEL FOR CANCER STEM CELL STUDY

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by

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Abbreviation	Description
AACR	American Association for Cancer Research
AKT	Protein kinase B
ALDH	Aldehyde dehydrogenase
AMP	Adenosine monophosphate
αMEM	Alpha minimal essential medium
ABC	ATP-binding cassette
ALCAM	Activated leucocyte adhesion molecule
AML	Acute myeloid leukemia
APC	Adenomatous polyposis coli
APS	Ammonium persulfate
ATP	Adenosine triphosphate
BCRP	Breast cancer resistance protein
BER	base excision repair
BSA	Bovine serum albumin
CAL 27	Human tongue squamous cell carcinoma cell (ATCC® CRL-2095^{TM})
CAL 27-Y cells	CAL 27 cells of 5th passage and below
CAL 27-O cells	CAL 27 cells of 6th -20th passage
CAM	Cell adhesion molecules
CaMKII	Calcium/calmodulin-dependent kinase II
CCND1	Protein coding gene for cyclin D1
cDNA	Complementary DNA
CD34	Hematopoietic progenitor cell antigen
CD45	Lymphocyte common antigen

CD105	Endoglin
CD166	Activated leucocyte cell adhesion molecule (ALCAM)
CDK	Cyclin-dependent kinase
CFU	Colony forming unit
CK-1	Casein kinase-1
CO_2	Carbon dioxide
CSC	Cancer stem cell
DAB	3,3'-Diaminobenzidine
DCS	Dialysed C-serum fraction
DEPC	Diethylpyrocarbonate
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide
DPSC	Dental pulp stem cell
Dvl	Dishevelled
ECL substrate	Enhanced chemiluminescent substrate
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EpCAM	Epithelial cell adhesion molecule
EMT	Epithelial-mesenchymal transition
ES cell	Embryonic stem cell
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum

FGF-2	Fibroblast growth factor 2
FGFR2	fibroblast-growth-factor receptor-2
FITC	Fluorescein isothiocyanate
FSP1	Fibroblast-specific protein-1
Fzd	Frizzled
GDP	Guanosine diphosphate
Gli1	GLI family zinc finger 1
GPCR	G-protein coupled receptor
GSK-3	Glycogen synthase kinase-3
GTP	Guanosine triphosphate
HA	Hyaluronan
HAS	Heat-stable antigen
HBSS	Hank's Balance Salt Solution
HCl	Hydrochloric acid
HGF	Hepatocyte growth factor
HIF-1	Hypoxia-inducible factor-1
HLA	Human leucocyte antigen
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papillomavirus
HSC	Hematopoietic stem cell
Hs27	Human foreskin fibroblast cell (ATCC® CRL-1634^{TM})
H_2O_2	Hydrogen peroxide
IARC	International Agency for Research on Cancer
IgG	Immunoglobulin G
IL-6	Interleukin-6

iPS cell	Induced pluripotent stem cell
ISCT	International Society for Cellular Therapy
JAK-STAT	Janus kinase–signal transducer and activator of transcription
JNK	c-Jun N-terminal kinases
LC_{50}	Lethal concentration 50
LEF	Lymphoid enhancer factor
LRP5/6	Low density lipoprotein related proteins 5 or 6
MACS	magnetic activated cell sorting
MAP	Mitogen activated protein
MDR	Multidrug resistance
MET	Mesenchymal-epithelial transition
MF	Microfilaments
MMP	Matrix metalloproteinases
M-PER	Mammalian protein extraction reagent
MRP	Multidrug resistance-associated protein
MSC	Mesenchymal stem cell
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide)
mTOR	Mammalian target of rapamycin
MYC	Protein coding gene for c-Myc transcription factor
NAD(P)	Nicotinamide adenine dinucleotide phosphate
NER	Nucleotide excision repair
NFAT	Nuclear factor of activated T cells
NFκB	Nuclear factor κB
NLK	Nemo-like kinase
NOD/SCID	Non-obese diabetic /severe combined immunodeficiency

OSCC	Oral squamous cell carcinoma
PBS	Phosphate buffer saline
PCP	Planar cell polarity
PCR	Polymerase chain reaction
PE	Phycoerythrin
PI3 kinase	Phosphatidylinositol-3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
POU	Pit-Oct-Unc transcription factor
pRb	Retinoblastoma protein
PVDF	Polyvinyl difluoride
RNA	Ribonucleic acid
ROCK	Rho-kinase
ROR	Receptor tyrosine kinase-like orphan receptor
rpm	Revolutions per minute
RTK	Receptor tyrosine kinase
RYK	Atypical receptor tyrosine kinase; Receptor-like tyrosine kinase
SC	Stem cell
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
Ser	Serine
siRNA	Small interfering RNA
SP cell	Side population cell
TBS	Tris buffered saline
TBST	Tris buffered saline -Tween® 20 buffer

TCF	T-cell factor
TEMED	Tetramethylethylenediamine
TGF-β	Transforming growth factor beta
Thr	Threonine
TIC	Tumour initiating cell
TLE	Transducing-like enhancer proteins
Tm	Melting temperature
TPC	Tumour propagating cell
TP53	Protein coding gene for tumour protein p53
VEGF	Vascular endothelial growth factor

PENCIRIAN SEL TITIS SKUAMOUS KARSINOMA ORAL CAL 27 SEBAGAI MODEL UNTUK KAJIAN SEL STEM KANSER

ABSTRAK

Peratusan sel stem kanser (CSC) agak rendah di kalangan populasi sel barah. Oleh itu, langkah-langkah pengasingan dan pengayaan CSC telah menjadi rutin bagi kerja-kerja penyelidikan yang melibatkan CSC. CAL 27 (ATCC® CRL-2095 TM) yang berasal daripada tisu lidah manusia, merupakan salah satu titisan sel epitelium skuamus karsinoma yang sering digunakan. Tujuan kajian ini dijalankan adalah untuk mewujudkan satu model titisan sel yang bersesuaian untuk mengkaji perkembangan CSC secara cekap, dengan mengurangkan masa bagi proses pengasingan dan juga langkah pengayaan sel. Kajian dijalankan untuk menentukan transformasi morfologi dan juga fisiologi sel titis CAL 27, terutamanya berkenaan dengan pengekspresian penanda sel stem mesenkima, tempoh masa penggandaan sel, rintangan terhadap rawatan, kebolehbalikan fenotaip, ciri invasif dan keupayaan metastatik. Selain itu, penglibatan lekatan sel dan peralihan epitelium-mesenkima (EMT), bersama dengan isyarat laluan Wnt juga diselidik. Hasilnya, pemanjangan sel CAL 27-O (sel CAL 27 dari generasi ke-6 dan seterusnya) dari sel CAL 27-Y (sel CAL 27 sebelum generasi ke-6) dikaitkan dengan peningkatan keupayaan invasif dan metastatik, rintangan rawatan yang lebih tinggi, pembalikan fenotaip yang mencirikan sel-sel epitelium, selain peningkatan kadar pembaharuan sel. Tambahan pula, sel yang mengekspreskan penanda positif sel stem mesenkimia (CD105) telah meningkat lebih daripada 70%. Walaubagaimanapun, pengekspresian sel dengan protein lekatan sel (CD166) telah menyusut sekurang-

kurangnya 90%, diiringi oleh peningkatan ekspresi vimentin dan penyusutan ekspresi E-cadherin untuk EMT. Selain itu, transformasi fenotaip didapati berkaitan dengan penyusutan aturan bagi laluan kekutuban sel satah (PCP) Wnt bukan kanonikal dalam sel CAL 27-O. Oleh itu, kajian ini boleh digunakan dalam kajian berkaitan CSC, untuk memahami perkembangan CSC dan juga untuk proses saringan dadah yang berpotensi untuk mensasarkan CSC.

CHARACTERIZATION OF CAL 27 ORAL SQUAMOUS CARCINOMA CELL LINE AS A MODEL FOR CANCER STEM CELL STUDY

ABSTRACT

The percentage of cancer stem cells (CSCs) is relatively low in tumour bulk population. Therefore, isolation and enrichment steps for CSCs have become routine for research works involving CSC. CAL 27 (ATCC® CRL-2095™) is one of the commonly used squamous epithelial carcinoma cell lines derived from human tongue tissue. The present study was conducted with the objective to establish a suitable cell line model for studying CSC development efficiently, by reducing the time consuming isolation and enrichment steps. Experiments were carried out to determine the morphological as well as physiological transformations of CAL 27 cell line, particularly on the expression of mesenchymal stem cell markers, cell doubling time, treatment resistance, phenotype reversibility, invasiveness and metastatic ability. In addition to that, involvement of cell adhesion and epithelial-mesenchymal transition (EMT), together with Wnt signaling pathway were investigated. As for the results, elongation of CAL 27-O cells (CAL 27 cells from 6th passage onwards) from CAL 27-Y cells (CAL 27 cells below 6th passage) were found to be associated with enhanced invasive and metastatic capability, greater resistance to treatment, reversal of phenotype characterizing epithelial cells, besides enhanced proliferation. Moreover, cells expressing mesenchymal stem cell positive marker (CD105) was increased for more than 70% with the transition. Contrasting to this, cells with cell-cell adhesion protein (CD166) was reduced by more than 90%, accompanied by overexpression of vimentin and downregulated expression of E-cadherin for EMT. Besides, the phenotypic transformations were

associated with downregulation of Wnt noncanonical planar cell polarity (PCP) pathway in CAL 27-O cells. Hence, the study could be extended in CSC related study, to understand the development of CSCs and also for screening of potential drugs targeting CSCs.

CHAPTER 1

INTRODUCTION

Abnormal growth of cells generates tumours. Benign tumours are not harmful to body tissues. Hence, only malignant tumours are cancers. Malignant cancers refer to the abnormal growth of cells that damage or destroy normal body tissues. Classification of cancer types is based on the types of cell that is initially affected. The abnormal growth of cells harms the body as the uncontrollable cell division interferes with the digestive, nervous and circulatory systems, except in leukemia where cancer prohibits normal blood function.

Oral cancer is the most common site of malignancy in the head and neck region that affect lips, tongue, gums, cheeks, sinuses and pharynx. Most of the patients with oral cancer are diagnosed at advanced stage that causes therapeutic complications with high mortality rate. The factor accountable for the late diagnosis is that the early and curable lesions are usually manifested with some common symptoms such as swelling lumps, velvety patches, and persistent soreness.

The overall survival rate differs significantly between the carcinoma stages, decreases from 75-90% for Stage I to 10-22% for stage IV. High frequency of loco-regional recurrences is often associated with oral cancer, which is implicated for most of the post-surgery and radiotherapy treatment failures. Relapsed oral cancer undoubtedly denotes a tough challenge for clinical practitioners due to the aggressiveness and invasive behaviours (Da Silva *et al.*, 2012).

The time course of relapse manifestation and metastatic progression are unpredictable. Currently, chemotherapy remains as the only option of treatment for relapsed cancer whenever surgery or re-irradiation is not possible. However, development of drug resistance in cancer cells has limited the efficacy of the chemotherapy treatment (Da Silva *et al.*, 2012). The high probability of oral cancer recurrence is frequently linked to ipsilateral or bilateral lymph node metastasis as the extensive lymphatic submucosal network enables the spreading of cancer cells to other site in head and neck region (Fan *et al.*, 2011).

Cancer cell populations are heterogeneous in which the self-proliferative population is known as cancer stem cell (CSC). According to American Association for Cancer Research (AACR), CSCs are defined as a small subset of cancer cells that make up a pool of self-sustaining cells with the special ability to self-renew and generate heterogeneous lineages of cancer cells (Clarke *et al.*, 2006). CSC population was first isolated by Bonnet and Dijk (1997) and Al Hajj (2003) in acute myeloid leukemia and solid tumours, respectively. The research obtained attention from many researchers ever since then.

Chemotherapy drugs that target the tumour bulk are only able to eliminate the chemotherapy sensitive non-CSC, while enriching the chemotherapy-resistant CSC and hence worsen the case by promoting recurrence (Frame and Maitland, 2011). Characteristics such as high proliferative ability, invasiveness, migration capacity, treatment resistance and phenotype reversibility are known to associate with CSCs (Reya *et al.*, 2001; Wicha *et al.*, 2006; Sayed *et al.*, 2011; Cabrera *et al.*, 2015; Hong *et al.*, 2015). These research findings indicated that CSCs are responsible for cancer

recurrence, treatment resistance and metastatic dissemination. The advancement of aggressiveness observed from relapsed oral cancer is parallel with the development of CSC.

Gioanni and colleagues established two new cell lines from squamous cell carcinomas of the tongue in 1982. One of the cell lines that was established was CAL 27 derived from a 56-year-old Caucasian male. The tumorigenicity of CAL 27 cells was confirmed in nude mice during cell line establishment. The cell line was established without any genetic manipulation (Gioanni *et al.*, 1988).

Ever since then, CAL 27 cell line was widely used to build oral squamous cell carcinoma (OSCC) model for *in vitro* and *in vivo* studies, and thus considered as a representative cell line for OSCC study (Jiang *et al.*, 2009). Based on CSC hypothesis, CSCs in CAL 27 cell line are responsible for the enhanced growth regulation, treatment resistance, phenotype plasticity, as well as invasiveness and metastatic ability of the cell line. Therefore, CAL 27 cell line was selected for this study.

1.1 Problem Statement

New findings involving CSC have been greatly discussed which brings about new ideas and directions for research. However, one of the challenges in CSC research is that the percentage of CSC population in tumour bulk is relatively low compared to non-CSC (Beck and Blanpain, 2013). Therefore, the process of identifying, isolating and enriching CSCs has always been a time-consuming process for the study. Currently, there is no such cellular model available to be used. In view

of this, a cellular model mimicking CSC development using currently available cell line could be a practical approach for screening study involving CSC.

The first part of this study focused on the morphological transformation of the cells, along with detecting and identifying CSC population from the bulk population of cells, using appropriate markers. Comparison on mesenchymal stem cell marker expression was performed against stem cells from the same region, dental pulp stem cells (DPSC). The second part of the study was directed towards characterization of the cancer-origin cell line according to CSC properties to recognize the presence and transformation of CSC in the cell line.

In the third part of the study, concurrent investigation on epithelial-mesenchymal transition (EMT) and cell adhesion proteins were done to provide an insight on the cellular regulation during CSC development. With these, investigation to reveal the missing puzzle on the signaling pathway(s) underlying CSC development, particularly related to the transformation of cell line, was performed in the last part of the study.

1.2 Rationale of study

Based on the understanding on CSC concept, identifying CSC from cancer-origin cell line is feasible. The CSC cellular model would be a more time-effective alternative for CSC research by reducing the time requirement for isolating and enriching CSCs for fundamental studies, as well as drugs screening. The model could be used to predict the development of CSC under defined circumstances created by researchers to understand carcinogenesis progression. Furthermore, the cellular

model can also be used to evaluate the efficiency of cancer treatment targeting CSC specifically.

1.3 Objectives

The general objective of this project is to establish a better-studied cellular model for CSC study by using CAL 27 cell line. In order to achieve the general objective, the following detailed objectives are defined:

1. To identify the morphological transformation of CAL 27 cells.
2. To identify and quantify the mesenchymal stem cell marker CD105 expressing population among CAL 27 cells.
3. To characterize cancer stem cell properties exhibited by CAL 27 cells.
4. To investigate on the protein regulation for cell adhesion and epithelial-mesenchymal transition (EMT) in CAL 27 cells.
5. To understand and propose a potential signaling pathway(s) involved in early cancer development.

CHAPTER 2

LITERATURE REVIEW

2.1 Cancer: The Killer Disease

Cancer has been so frequent that every one of us would have at least a relative or friend that was involved in fighting cancer. According to the last Health Facts 2013 from Ministry of Health (MoH) Malaysia, cancer is one of the top ten reasons of hospitalisation and one of the top five causes of death. The most frequently encountered cancer types among Malaysians are breast, colorectal and lung cancer.

Based on reports from the International Agency for Research on Cancer (IARC) Globocan of the World Health Organisation reported by Ferlay *et al.* (2013), one out of 19 Malaysians develops breast cancer, one in 33 develops colorectal cancer and one in 40 develops lung cancer. In Malaysia, the number of cancer incidence increased from 32,000 new cases in 2008 to about 37,000 in 2012. There were only 21,100 cancer mortality in 2008, but the figure has increased to 21,700 deaths in 2012.

In 2012, 14 million people were diagnosed with cancer worldwide, according to the World Cancer Report 2014, a new global cancer report compiled by IARC. The striking fact reported in the same report is that cancer has overtaken heart disease as the number one killer in 2014. To reduce the number of cases, there is an accelerated demand for better understanding on cancer development for the most effective and efficient ways to control cancer.

2.2 Cancer Progression

Cancer can begin from any cell in the body. These cells start out as normal cells, but then changes with exposure to mutations and become neoplastic. The development of cancer is a multistep process in which cells gradually become malignant through a progressive series of alterations, indicated by development of cancer late in life. Mutations affect how cells grow, work, reproduce and die. They may cause the cell to continue growing and dividing uncontrollably instead of dying when it is the appropriate timing. Along the expansion of cancer cell population, random mutations occur in the individual cells, leading further to divergence in molecular identity (Korbel and Campbell, 2013).

The process of cell changes in which a cell loses its capacity to manage its rate of division, and thus becomes a cancer cell, is known as cell transformation. Although there are many different types of cancer, they all start because of uncontrolled and abnormal growth of cells. The cancerous cell generally retains the structural and functional characteristics of the normal cell type from which it is derived. Almost 80% of all cancers were epithelial cancers from breast, colon, prostate and ovary tissues (Diaz-Cano, 2012).

The typical characteristics of cancer are often referred to the poorly differentiated phenotype, and the genetic and functional heterogeneity. Within tumours, functional heterogeneity was observed as cells exhibited variable proliferative and differentiation capacity. While environmental and other non-genetic factors are involved in tumorigenesis, it is commonly accepted that cancer occurs because of mutations in cancer-susceptible genes namely oncogenes and tumour

suppressor genes. Both oncogenes and tumour suppressor genes are gate-keepers in cancer initiation (Pizon *et al.*, 2013).

Oncogenes are damaged version of normal genes, the proto-oncogene. These proto-oncogenes control various cell functions, particularly in cell growth and reproduction. When proto-oncogenes are mutated or activated to turn into oncogenes, cells grow out of control and the transformation promotes cancer cell growth. Oncogenes have been discovered to take part in different stages of human cancers-tumour initiation, progression, angiogenesis and metastasis (Shortt and Johnstone, 2012).

On the other hand, tumour suppressor genes are the genes work to guard against cancer. These genes act as barriers that help to stop cell growth and regulate cell death such as apoptosis. However, if the tumour suppressor genes are damaged or missing, their implementations on cell proliferation and cell death are unchecked. Thus, the alteration leads to cancer progression. For example, TP53; the tumour suppressor gene that prompts cell death, is often found to be damaged or missing in several types of cancer (Kitagishi *et al.*, 2012).

Progressive changes of human cancer from pre-malignant lesions to fully malignant invasive tumours have been well recognized from the recorded cases. Accumulation of genetic alteration converts pre-malignant cells into more aggressive malignant cells, producing primary tumour. At the early stage of primary tumour expansion, the cells are not invasive or metastatic. Due to the genetic instability in the cells, further genetic alterations occur. Thus, new clones with invasiveness and metastatic capacity are produced, which are the fully malignant cells. However, only

a fraction of the cells in primary tumours acquire the invasive and metastatic ability, creating heterogeneity in the cell populations (Diaz-Cano, 2012).

2.3 Types of Cancer

Cancer can begin from abnormal proliferation of any of the cells in the body. Therefore, there are more than a hundred distinctive cancer types, which can vary considerably in their aggressiveness and response to treatment.

The most noticeable issue in cancer pathology is the difference between benign and malignant tumours. A benign tumour remains restricted to its original location, but not invading the surrounding normal tissue or spreading to other body sites. In contrast, a malignant tumour is capable of both invading surrounding normal tissue and spreading throughout the body via the circulatory or lymphatic systems, a condition which is known as metastasis. Cancers, which are commonly referring to those malignant tumours, are dangerous due to their ability to invade and metastasize. Benign tumours can usually be removed by surgery, but the spread of malignant tumours to distant body sites often renders effectiveness of localized treatment to these tumours (Valastyan and Weinberg, 2011).

Classifications of both benign and malignant tumours are based on the type of cell from which they arise. The three main categories for most of the cancer types are carcinomas, sarcomas, and leukaemias or lymphomas. Approximately 90% of human cancers are carcinomas, developing from epithelial cells. Sarcomas are solid tumours of connective tissues such as muscle, bone and cartilage. Leukemias and lymphomas arise from the blood-forming cells and from cells of the immune system respectively,

occupy approximately 8% of human malignancies. Tumours are further classified according to their tissue of origin, for example lung or breast carcinomas (American Cancer Society, 2011)

2.4 Oral Carcinoma

95% of head and neck cancer cases are diagnosed as head and neck squamous cell carcinoma (HNSCC) that occur in over half a million people globally. HNSCC is the sixth most common malignancy in the world (Sayed *et al.*, 2011). The prognosis for patients with recurrent or metastatic HNSCC is generally poor with low 5-year survival rates despite treatment advances over the past few decades, with surgery and radiation being the core treatment method (Suma *et al.*, 2015).

Approximately half of those diagnosed cases of HNSCC occur specifically in the oral cavity, reported as oral squamous cell carcinomas, which might involve lip, tongue, gum, cheek, floor of mouth, sinuses and pharynx. The symptoms detected such as velvety patches, swelling, lumps or bumps in the oral region with persistent soreness can be easily mistaken as other infectious diseases (Tamashiro *et al.*, 2013).

The factors leading to oral cancer include not only alcohol and tobacco that were known to be the traditional ones. However, there has been increasing number of incidence reported in young patients which are caused by betel quid chewing (Guha *et al.*, 2014) and human papillomavirus (HPV). Oral cancer cases developed from HPV infection is a factor discovered recently, especially in the United States (Martin-Hernan *et al.*, 2013).

Despite recent advances in cancer detection and management, most of the patients present with late-stage disease because the clinical signs and symptoms of head and neck tumours are often nonspecific and tend to be mistaken for other common ailments. Consequently, it is essential to search for new biomarkers and effective therapy options to optimize cancer treatment (Martinez-Useros and Garcia-Foncillas, 2015).

2.5 Properties of Cancer Cells

Losing control over growth, cancerous cells are formed as a result of accumulated aberrations that interfere with many of the regulatory mechanisms. With the interference from these unregulated mechanisms, the reflected cell behaviour display abnormalities in cell proliferation, survival and differentiation that distinguish cancer cells from the normal counterparts. These properties serve as a characterizing description for cancer cells (Hanahan and Weinberg, 2011).

Cancer cells have reduced requirements for extracellular growth factors as compared to normal cells. Proliferation of most of the cells in our body is controlled, mostly by polypeptide growth factors. For certain cell types, the availability of serum growth factors is the key determinant for their proliferative capacity in culture. This property is particularly important in fibroblasts. The requirement for growth factors of cancerous cells is closely related to density-dependent inhibition, since the cell density is the key determinant for cells to enter quiescent stage (Yang and Xu, 2011).

Cancer cells are able to produce growth factors that stimulate their own proliferation in certain cases. Production of growth factors by a cancer cell can lead

to continuous autocrine growth stimulation that stimulates cell division in the niches. In this condition, cancer cells are less dependent on growth factors from other sources. Also, the reduced growth factor dependence of cancer cells is a result from aberrations in intracellular signaling systems, such as upregulation or hyperactivity of growth factor receptors or other proteins (Ras proteins or protein kinases) (Witsch *et al.*, 2010).

Conversely, cancer cells secrete growth factors that promote the formation of new blood vessels in angiogenesis. Angiogenesis is needed to support the tumour outgrowth that grows beyond the size of normal cells. With increased requirement to oxygen and nutrients supply, new blood vessels are formed to support the need. In metastasis, the actively growing new capillaries formed are easily penetrated by the tumour cells, providing a ready opportunity for cancer cells to enter the circulatory system and begin the metastatic process (Witsch *et al.*, 2010).

As illustrated in Figure 2.1, normal cells encounter density-dependant inhibition during cell growth. During proliferation, normal cells divide and multiply until they reach certain density. The multiplication stops proliferating and arrested in G_0 stage of cell cycle to remain quiescent. The key distinction between cancer cells and normal cells in culture is that the cancer cells are unresponsive to density-dependent growth inhibition. Cancer cells proceed to continue growing towards high cell density in culture, mimicking their uncontrolled proliferation *in vivo* and in real case scenario (Leontieva *et al.*, 2014; Reece *et al.*, 2013).

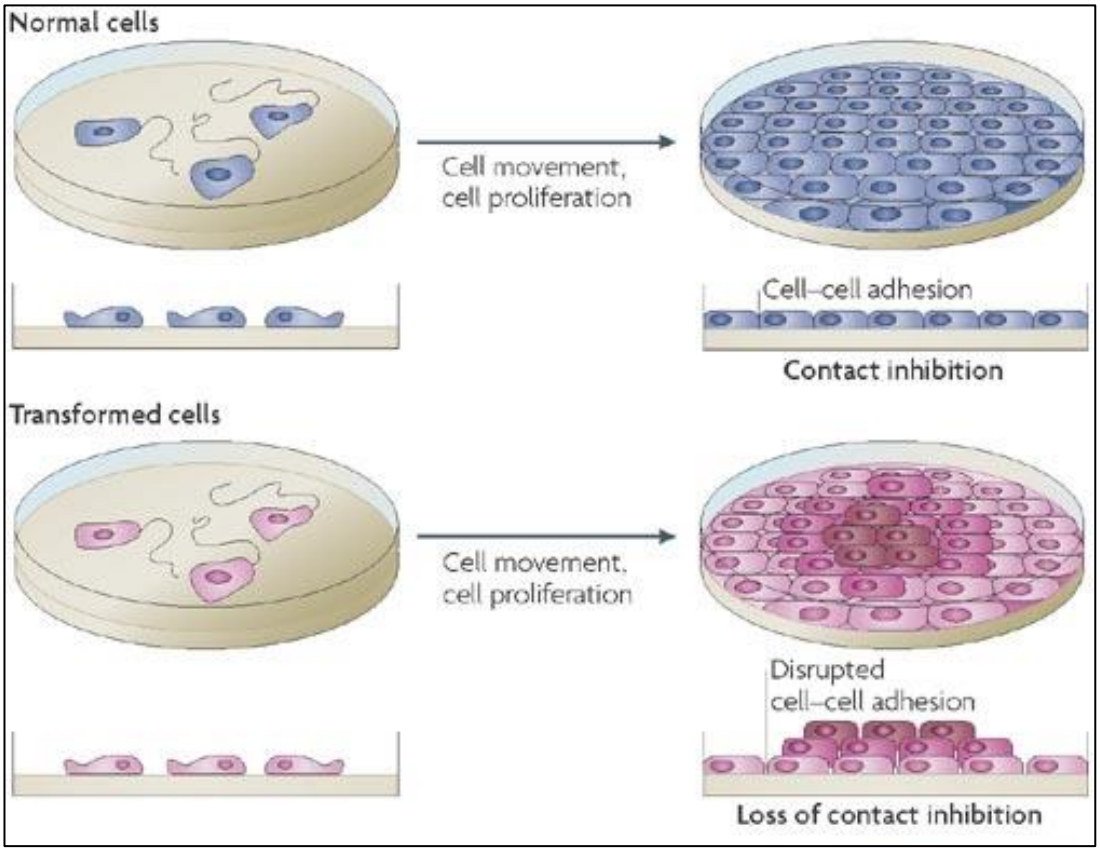

Figure 2.1: Density-dependant inhibition. Normal cells proliferate in culture until they reach a finite cell density coming into contact with other cells, at which point they become quiescent. Tumour cells, however, continue to proliferate independent of cell density. The concepts of 'contact inhibition of cell proliferation' and 'contact inhibition of cell movement' are often taken as two sides of the same coins.

(Takai *et al.*, 2008)

Cancer cells are not restricted by contact inhibition as opposed to normal cells. Normal fibroblasts migrate across the surface of a culture dish until they come into contact with a neighbouring cell. Further cell migration is inhibited that causes the normal fibroblast cells to adhere to each other, forming an organized arrangement of cells on the culture dish surface. Instead, cancer cells continue moving after contact with their neighbours, migrating over neighbouring cells. The growth is in a disordered, multi-layered pattern. Cancer cells are characteristically insensitive to such contact inhibition of growth and movement (Kim and Asthagiri, 2011; Batson *et al.*, 2013).

Cancer cells are different from normal cells in their cell-cell and cell-matrix interactions. Most cancer cells are less adhesive than normal cells, due to reduced expression of cell surface adhesion molecules. For example, loss of E-cadherin in development of carcinomas (epithelial cancers), the key adhesion molecule has been observed. As a consequence of reduced expression of cell adhesion molecules, cancer cells are able to invade and metastasize to other tissues with less restricted interactions with other cells and tissues. The reduced adhesiveness of cancer cells also leads to morphological and cytoskeletal alterations (Jung *et al.*, 2012).

In the microenvironment, cancer cells exhibit additional properties that empower them in invasion and metastasis. Malignant cells secrete proteases that digest extracellular matrix components, allowing the cancer cells to invade adjacent normal tissues. For instance, secretion of collagenase aids in digestion and

penetration of malignant cells through basal laminae to invade underlying connective tissue in carcinoma development (Lu *et al.*, 2011; Man *et al.*, 2013).

Most cancer cells display defective differentiation, which is closely related to abnormal proliferation. Fully differentiated cells cease cell division or seldom divide. Instead of normal differentiation, cancer cells are usually blocked at the stage of specialization, corresponding to their continued active proliferation. One of the examples to show the relationship between defective differentiation and malignancy can be observed in leukaemia. Leukemic cells fail to undergo terminal differentiation in generation of erythrocytes, lymphocytes, granulocytes, or macrophages. They remained to be at early stages of development at which they retain their ability for proliferation (Diaz-Cano, 2012).

In addition to that, many cancer cells fail to undergo apoptosis, the programmed cell death. As a result, the life spans of cancer cells are longer in comparison to normal cells. Failure of cancer cells to undergo apoptosis enables cancer cells to survive in condition deprived of growth factors from environmental signals, contrasting the survival of normal cells which rely on signal from growth factors or extracellular matrix that prevent apoptosis. Instead of undergoing apoptosis with DNA damage, cancer cells fail to correct the situation. Being non-responsive to apoptosis, cancer cells become resistant to radiation and chemotherapeutic drugs in treatment (Su *et al.*, 2015).

2.6 Stem cells at the top of hierarchy

Stem cells have been a hot topic in biological science. The stem cells are unique due to their unlimited regenerative potential to produce more stem cells and tissue specific differentiation capacity to differentiate into diverse specialized cell types. With more research activities being involved, stem cell technology would be the trend for treatment for diseases (Duran and George, 2011).

Categorisation of stem cells can be based on the potency of the cells. Totipotent stem cells differentiate into embryonic and extraembryonic cell types to give rise to a fully functional organism, as well as to every cell type of the body. Pluripotent stem cells are derived from totipotent stem cells, can give rise to almost any cell types from any of the three germ layers or any cell types in adult organism. Embryonic stem cells are in this category (Horie *et al.*, 2011; Hima and Srilatha, 2011).

Multipotent stem cells are more differentiated cells, which mean that their possible lineages are less variable or more determined. Thus, multipotent stem cells are only capable to give rise to a limited number of cells from closely related family. Adult hemapoietic stem cells and MSCs are in this category. For example, MSCs have been shown to produce bone, muscle, cartilage, fat, and other connective tissues. These tissue specific stem cells are indispensable for maintaining tissue homeostasis and repair (Hima and Srilatha, 2011).

There are many prospective sources where stem cells can be obtained. Embryonic stem (ES) cells are derivatives from the inner cell mass of a blastocyst,

whereas fetal stem cells can be found in fetal organs. Cell therapy using stem cells introduce stem cells to targeted site of tissues to treat diseases with or without gene therapy. Pluripotent ES cells have been isolated from inner cell mass of early embryos for the almost limitless potency in biological research that is able to generate mostly all cell types. However, the promising research raised ethical issues, leading the development of induced pluripotent stem (iPS) cells. One key drawback of stem cell therapy using ES and iPS cells is on their potential for teratoma formation, compromising their application in regenerative medicine (Wei *et al.*, 2013).

Cells have to be replaced even after complete maturation of an organism. Adult stem cells have been isolated from various tissues, including central nervous system, bone marrow, retina, skeletal muscle and dental pulp (Hima and Srilatha, 2011; Abdullah *et al.*, 2013). Stem cells play their role in internal repair system in most of the tissues, with their capability to divide and replenish damaged cells. The new cells after division can be channelled into different differentiation path to become more specialized cell types such as muscle cells, red blood cells and white blood cells. Hematopoietic stem cells (HSCs) have been widely used for allogeneic cell therapy, meaning that the donor is different from the recipient of the cells. (Hima and Srilatha, 2011).

With the new hope generated from application of mesenchymal stem cells (MSCs) in biomedical field, MSCs catch the attention of scientists for more investigations. However, investigations using different isolation and expansion methods created ambiguities and inconsistency as it is difficult to compare and

contrast study outcomes without a standard method to characterize MSC. To address the issue, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) proposed to set the minimal criteria to define human MSC (Dominici *et al.*, 2006).

The first criterion being set by ISCT is that the cells must exhibit adherence to plastic in standard culture conditions. Secondly, MSC must express CD105, CD73 and CD90 (≥ 95% of cell population), but not CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR (≤ 2% of cell population). Thirdly, these cells must be able to differentiate into osteoblasts, adipocytes and chondroblasts as demonstrated by staining of *in vitro* cell culture. This minimal set of criteria standardise a uniform characterization of MSC, so that exchange of data among researchers in the field becomes relevant. These criteria, particularly plastic adherence and marker expression, were applied in this study for characterizing stem cells (Dominici *et al.*, 2006).

Stem cells isolated from adult dental pulp are termed as dental pulp stem cells (DPSCs). DPSCs can be collected from dental pulp by means of a non-invasive method that can be performed in adults using simple surgical wisdom teeth extraction, a routine surgical practice with less ethical issues. DPSCs have been studied for the ability to develop into active neurons, osteoblasts precursors, as well as chondrogenic and myogenic differentiation potential (Abdullah *et al.*, 2013). In this study, DPSCs were used as a comparison control for stem cell characteristics as there is a close proximity between DPSCs and oral carcinoma concerning tissue of origin, and also concerning debates on ethical issues in stem cells research.

On the other hand, stem cells have been used in replenishment of blood and immune system suffering from damage during cancer treatment by chemotherapy or radiotherapy. Other than immune-reconstitution, stem cells particularly MSCs have been reported to be used in cell-based bone reconstitution following chemotherapy and surgery in malignancies due to their regeneration and differentiation capacity (Suma *et al.*, 2015).

2.7 Stem cells and CSCs

Scientists and oncologists have been working on a long-standing goal of understanding the development of cancer, so that a framework can be established for successful treatment. One of the frameworks that attracted much attention is to understand cancer as a perturbed mechanism arising from cells in normal tissues that retain the tissue specific developmental features (Diaz-Cano, 2012).

Furthermore, analysis on morphology and proliferation of stem cells leads to understanding of heterogeneity of stem cell population that not every cell in the population actively proliferates at the same time within a given tissue. With these observations in both normal and aberrant tissues, it has been hypothesized that the differentiated cells originate from the undifferentiated cells with regenerative ability (Beck and Blanpain, 2013).

Former research was led to focus on understanding the genetic changes that direct a cell towards aberration in development without considering which cells are affected by mutations. The most recent model for carcinogenesis is the “stem cell hypothesis”, in which CSCs are the sites of mutations that initiate malignancy. With

this model, cancer has been presented in a hierarchy structure where CSCs are at the top of pyramid to perform self-renewal to support on-going tumour growth and tissue-specific differentiation (Mannelli and Gallo, 2012; Suma *et al.*, 2015).

In fact, there are diverging opinions on the origin of CSCs. Some researchers supported the view that cancers are derived from naïve stem cells that retain self-renewal ability but accumulated mutations that lead to epigenetic and genetic changes for cancer development. On the other side, the opponents stated the possibility that CSCs can be derived from differentiated cells that undergo dedifferentiation and reactivate the self-propagating activity to cause malignancy (Clarke *et al.*, 2006; Van de Stolpe, 2013).

2.8 History of concept development

The concept started when pathologist Virchow (1855) suggested that tumour could be derived from embryonic cells. Following this, Bruce and colleagues (1963) showed that only 1-4% of transplanted murine lymphoma cells can form colonies *in vitro* or can initiate a carcinoma in mouse spleen. The concept of cancer cells acquired basic features of normal stem-like cells, such as proliferation potential and differentiation capacity was proposed by Pierce and Speers (1988).

Previous research by Bonnet and Dick (1997), it was revealed that a single leukemic cell that was selected and isolated based on cluster of differentiation marker (CD markers) for CSCs, was able to transmit the disease in mouse after transplantation. The first solid tumour from which CSCs were identified and isolated was from breast cancers by Al-Hajj and colleagues (2003), which were characterized

as a $CD44^{high}/CD24^{low}$ subpopulation, and positive for epithelial cell surface antigen. The 100 cells being selected using the specific phenotype were able to proliferate and form new tumours when transplanted in mice.

With these reports, more research works were initiated and reports on solid tumours were consecutively reported, as from Singh *et al.* (2003) on brain tumour, Collins *et al.* (2005) on prostate cancer, O'Brien *et al.* (2007) on colon cancer, Li *et al.* (2007) on pancreatic cancer, Quintana *et al.* (2008) on melanoma, Sell and Leffer (2008) on liver cancer. Nevertheless, there are still doubts and criticism over the existence of CSCs and their functional role in cancer progression (Maenhaut *et al.*, 2010). On-going research on CSCs are still being carried out to answer the questions that seems to get more complicated with more data accumulating. The postulations and deductions on CSCs are reviewed from time to time to be in pace with the latest research reports.

2.9 New era of cancer research-the cancer stem cell concept

Most cancers remain not curable with the currently available therapeutic approaches, based on the knowledge that we have learnt about cancer biology in the last decades. Current treatments are based on the assumption that cancer is a proliferation-based disease. According to this assumption, the main treatments are anti-proliferative and they are non-specific and with serious side-effects. Although cytotoxic drugs and radiation reduce tumour burden, relapse of the disease occurs in the majority of the cases (Pisco and Huang, 2015).

At least two models have been proposed to explain heterogeneity in cancer cells for the propagating properties and differentiation status: hierarchical cancer stem cell model as shown in Figure 2.2 and stochastic model. For each of the model, clonal evolution could happen as a result of interaction of microenvironment and cells, leading to accumulated epigenetic changes, as demonstrated in Figure 2.3 (Sayed *et al.*, 2011; Beck and Blanpain, 2013).

Cancer stem cell concept has been derived from understanding on normal stem cells. The issue of stem cells has been raised corresponding to cancer development. It was found that stem cells and tumour cells exhibit common features on their unlimited proliferative and tissue-specific differentiation capacity (Trosko and Chang, 1989). The unique replication process in normal tissues allows a stem cell to receive signals from the microenvironment to divide and expand the stem cell population (Morrison and Kimble, 2006).

According to the cancer stem cell model, a small population of cancer cells known as CSCs make up the reservoir of self-sustaining cells with their unique properties to propagate and differentiate into the bulk of tumours. CSCs have the capacity to divide as well as to expand the cancer stem cell pool and to differentiate into the heterogeneous non-tumorigenic cancer cell types to form the bulk of the cancer cells within the tumour (Nguyen *et al.*, 2012).

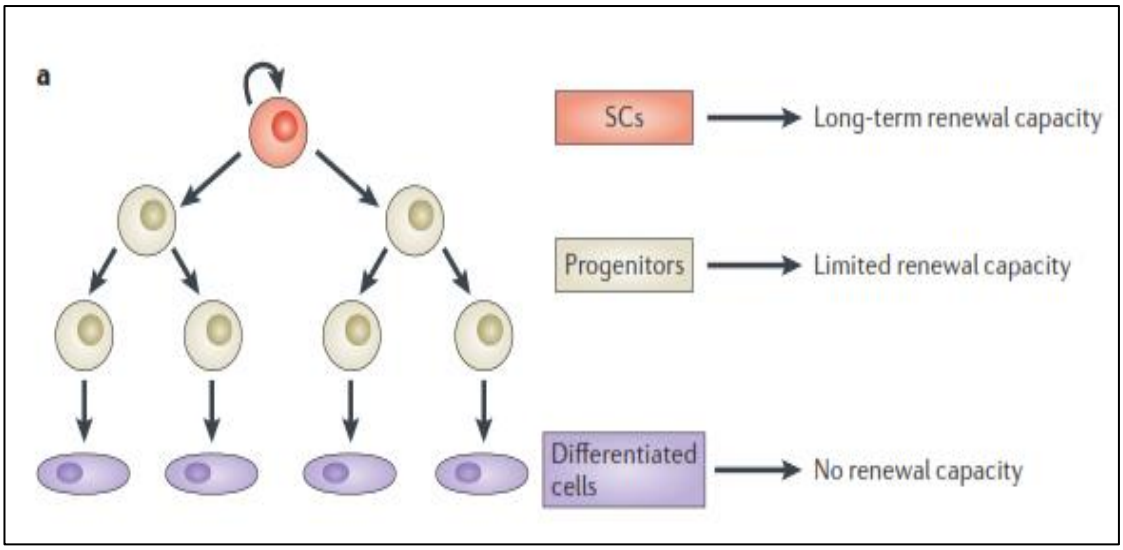

Figure 2.2: Hierarchy in tumours. The cellular hierarchy in tumours implies that not all cells are equivalent and that only CSCs present long term self-renewal and differentiation potential. CSCs give rise to new CSCs, as well as more committed progenitors with more restricted renewal potential which eventually produce terminally differentiated cells.

(Beck and Blanpain, 2013)

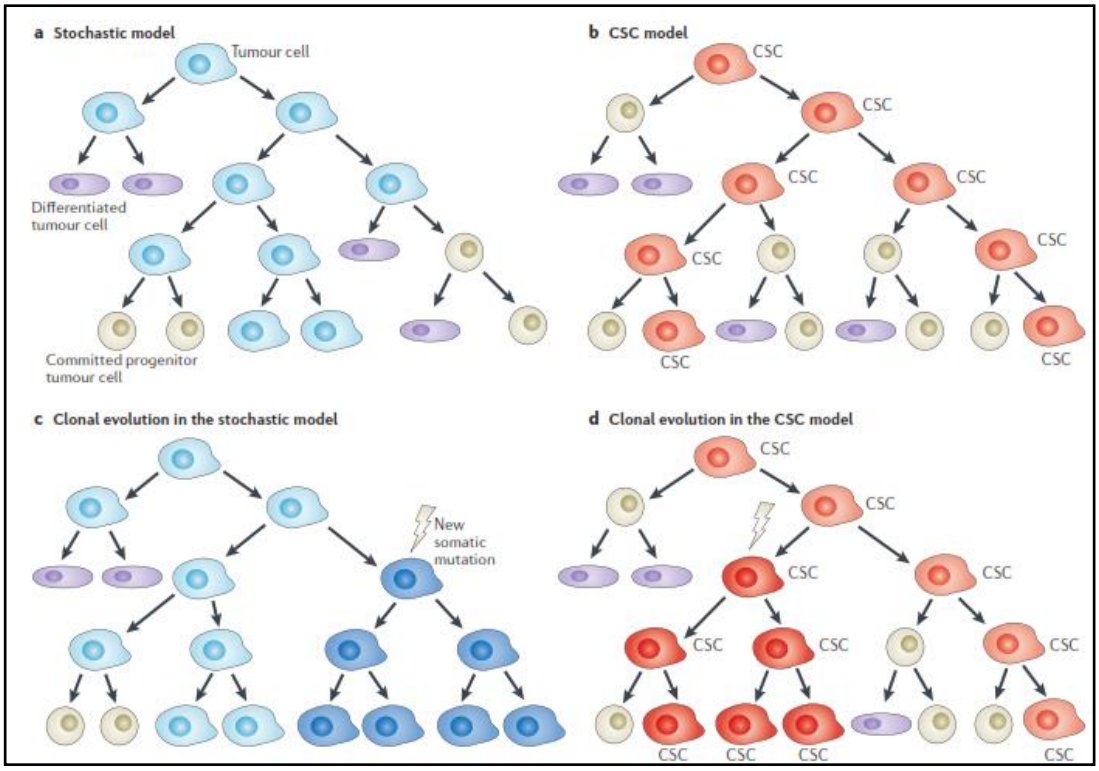

Figure 2.3: Clonal evolution in stochastic and cancer stem cell model

a. According to stochastic model, all tumour cells are equipotent and randomly self-renew to expand the population or differentiate to specific tissue.
b. With hierarchical CSC model, only a subset of tumour cells has the ability for long-term self-renewal and these cells give rise to progenitors with limited proliferative potential that eventually terminally differentiate.
c. Clonal evolution in stochastic model explains that tumour cells transform into more aggressive cell type with new somatic mutation.
d. Clonal evolution in CSC model describes the generation of clonal diversity with mutations, which further increase tumour heretogeneity.

(Beck and Blanpain, 2013)