

The isolation, characterisation and role of cancer stem cells in oral cancer progression

By

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Abstract

Introduction: Oral cancer has a poor prognosis due to late detection and the high risk of recurrence and development of secondary tumours at distant sites. This may be attributed to the fact that conventional anticancer therapy does not eradicate the cancer stem cells which are a subset of the tumour cell population characterised by unique properties including their capability for indefinite self-renewal, proliferation and differentiation to diverse heterogeneous cell lineages that form the bulk of the tumour. Moreover, they are highly tumourigenic, play a crucial role in invasion and metastasis and also have the ability to resist anticancer therapy. Identifying and isolating CSCs is considered an important step in order to study their biological characteristics accurately with the aim of subsequently targeting and eradicating them. CSCs isolation has previously been mainly based on the expression of specific markers or clonogenicity as well as the rapid adherence of such cells to ECM proteins.

Aims: The purpose of this study to develop functional assays (adhesion and chemoresistance) to isolate CSCs from OSCC cell lines and investigate the stem cell characteristics of the sorted cells compared to unsorted cells. Additionally, we aimed to investigate the stability of the isolated phenotypes and determine whether the CSCs signal differently from non-CSCs to stromal cells (fibroblasts).

Methods: Cancer stem cells were isolated from 2 different oral squamous cell carcinoma cell lines (H357 and SCC4) using 2 different functional assays on the basis of their rapid adherence to fibronectin (75µg/mL, 10 mins) and their resistance to conventional chemotherapy (15µM cisplatin for 24 hours). The stability of the early adherent phenotype was investigated by repeating the adhesion assay after incubation of the early adherent phenotype in culture for 48 hours. For the chemoresistant cells phenotype cells were treated with same dose of cisplatin for second time. The isolated cells by both functional assays were characterised by molecular assays (flow cytometry and qPCR) as well as functional assays (proliferation and colony forming assays). Moreover, the effects of CSCs on activation of normal oral fibroblasts (NOFs) into cancer associated fibroblasts (CAFs) was assessed by incubation of NOFs in low serum conditioned medium collected from both sorted and unsorted cells for 48 hours. The gene and protein expression of the activation markers alpha smooth muscle action (α -SMA) and interleukin-6 (IL-6) was then assessed using qPCR and

immunofluorescence. In addition, an immunohistochemical study was performed to investigate the *in vivo* correlation between CSC markers (CD24 and CD44) and stromal marker (α -SMA) in patient samples of oral squamous cell carcinoma.

Results: The isolated cells from both cell lines using both functional methods expressed highly significant levels of stem cell markers and stem associated genes (CD24, CD44 and CD29) compared to unsorted cells. In addition, the isolated cells showed low growth rate and high colony forming efficiency compared with unsorted cells. Moreover, the early adherent phenotype of the both tested oral cancer cell lines showed a continuation of rapid adherence to fibronectin after incubation in culture for 48 hours. Similarly, chemoresistant cells showed higher significant growth rate compared to unsorted cells following a second exposure to chemotherapeutic drug. The early adherent cells showed chemoresistance levels significantly higher than unsorted as well as chemoresistant cells being more rapidly adherent to fibronectin than unsorted cells. Expression of both α -SMA and IL-6 genes was increased in NOFs by 2-3 fold as a result of exposure to either H357 or SCC4 CSCs conditioned medium when compared to unsorted OSCC cells. Increased levels of α -SMA protein staining were also observed. Furthermore, immunohistochemical analysis revealed a positive correlation between the expression of CSC markers (CD24 and CD44) and an activated fibroblast marker (α -SMA) in the tumour microenvironment of oral squamous cell carcinoma patient samples.

Conclusions: Our data suggests that rapid adherence to fibronectin and chemoresistance to cisplatin effectively isolate the same sub-population of cells from cell lines which show stem cell like characteristics. Factors released by CSCs can specifically activate fibroblasts and the *in vivo* correlation between CSCs and activated stromal fibroblasts in oral squamous cell carcinoma suggests that one of the mechanisms by which CSCs drive tumour progression is through their activation to the fibroblasts in the tumour microenvironment.

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LIST OF ABBREVIATIONS

®	Trade mark
α	Alpha
α-SMA	Alpha-smooth muscle actin
β	Beta
μ	Micro
μg	Micro gram
μm	Micro meter
μM	Micro molar
μL	Micro litre
ABC transporters	ATP-binding cassette transporter proteins
ADAM	a disintegrin and metalloproteinase
ALDH	Aldehyde dehydrogenase
AP-1	Activating protein-1
BODIPY	Boron-dipyrromethene
BSA	Bovine serum albumin
Ca ²⁺	Calcium ion
CAF	Cancer associated fibroblast
CDK	Cyclin-dependent kinases
CHK	Checkpoint kinase
CSC	Cancer stem cell
cDNA	Complementary deoxyribonucleic acid
CO ₂	Carbon dioxide
Conc	Concentration
Ct	Cycle threshold
CXCL12	Chemokine (C-X-C motif) ligand 12
DMEM	Dulbecco's modified eagles medium
DNA	Deoxyribonucleic acid
DRPs	Damage recognition proteins
EBV	Epstein- Barr Virus
ECM	Extracellular matrix
EDTA	Ethylene diamine tetraacetic acid

EGF	Epidermal Growth Factor
ELISA	Enzyme-linked immunosorbent assays
EMT	Epithelial-mesenchymal transition
EndoMT	Endothelial-mesenchymal transition
Ep-CAM	Epithelial cell adhesion glycosylated membrane
ESC	Embryonic stem cells
EVs	Extracellular vesicles
FAP	Fibroblast activating protein
FACS	Fluorescence activated cell sorting
FAK	Focal adhesion kinase
FBS	Foetal bovine serum
FCS	Foetal calf serum
FGM	Fibroblast growth media
FGF	Fibroblast growth factors
FSP-1	Fibroblast specific protein-1
HGF	Hepatocyte growth factor
HH	Hedgehog ligand
HIV	Human immune deficiency virus
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papilloma virus
hr	Hour
HIF1 α	Hypoxia inducible factor 1- α
G-CSF	Granulocyte-colony stimulating factor
IC ₉₀	Inhibitory concentration 90
ICAM-1	Intercellular adhesion molecule-1
IGF-1	Insulin-growth factor-1
IFN- α	Interferon- α
IU	International unit
IL	Interleukin
L	Litre
KGM	Keratinocyte growth medium
M	Molar
MACS	Magnetic activated cell sorting
MCP1	Monocyte chemotactic protein-1

MET	Mesenchymal-endothelial transition
mg	milli gram
Mg ²⁺	Magnesium ion
miRNA	Micro Ribonucleic acid
min	Minute
mL	milli litre
mM	milli molar
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3- carboxymethoxyphenyl) -2-(4 sulfophenyl) 2H-tetrazolium, inner salt
MVB	multivesicular bodies
NF-K β	Nuclear factor kappa-light chain enhancer of activated B cells
ng	nano gram
nm	nano metre
NOF	Normal oral fibroblast
NG2	Neuron-Glial 2
°C	degree Celsius
OCCL	Oral cancer cell line
OD	Optical density
OH	Hydroxyl radical
OSCC	Oral squamous cell carcinoma
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
qPCR	Quantitative polymerase chain reaction
Rb	Retinoblastoma
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Reverse transcription
SASP	Senescence-activated-secretory-phenotype
SD	Standard deviation
SDF	Stromal derived factor
SFM	Serum free medium

SEM	Standard error mean
SC	Stem cell
SP	Side population
TAC	Transit amplifying cell
TGF- β	Transforming growth factor-beta
TNF- α	Tumor necrosis factor-alpha
v/v	Volume/volume
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor

CHAPTER 1

REVIEW OF LITERATURE

1.1 Normal stem cells (SCs)

Stem cells are uncommon, immortal cells that have the capability to preserve themselves by self-renewal as well as differentiation to specialized cells of a specific tissue (Reya *et al.*, 2001). They are present in various somatic tissues and are considered crucial contributors to the physiology of these tissues and the maintenance of homeostasis (Jordan, Guzman and Noble, 2006), through cell repair (Soltysova, Altanerova and Altaner, 2005).

1.1.1 Characteristics of stem cells

Stem cells have 3 distinguishing features that make them unique, which are:

1. The ability of self-renewal (in other words, during cell division at least one of daughter cells maintains the same biological properties of the parent cell).
2. The ability to differentiate to diverse cell lineages.
3. The capability to proliferate continuously (Jordan, Guzman and Noble, 2006).

1.1.2 Patterns of spatial ordering of stem cells and their progeny in oral epithelium

Tissue renewal of the epithelial layer that covers the oral mucosa depends mainly on the epithelial stem cells (Iglesias-Bartolome, Callejas-Valera and Gutkind, 2013) which are located chiefly in the basal cell layer of the stratified squamous epithelium. Therefore, cell division occurs primarily in this layer (Bacelli and Trumpp, 2012).

The function of stem cells in preserving the structure of the epithelium requires a proliferative hierarchy with a specific spatial ordering. In this hierarchal model the stem cell situated at the apex divide either asymmetrically or symmetrically (González-Moles *et al.*, 2013), as shown in figure (1.1). Asymmetrical division of stem cell will give rise to one stem cell and one transit amplifying cell (TAC), which has the ability to divide 3-5 times until all daughter cells terminally differentiate. The main function of the TACs is to increase the number of differentiated cells due to the limited self-renewal capacity of these cells in comparison with stem cells (González-Moles *et al.*, 2013).

On the other hand, symmetrical division of stem cells generates either two TACs accompanied by loss of the parent stem cell or two stem cells which retain properties

of the parent cell. However, symmetrical division only occurs during recovery of lost basal stem cells populations as a result of disease or trauma (Mackenzie, 2006) or in early embryonic development (Soltysova, Altanerova and Altaner, 2005).

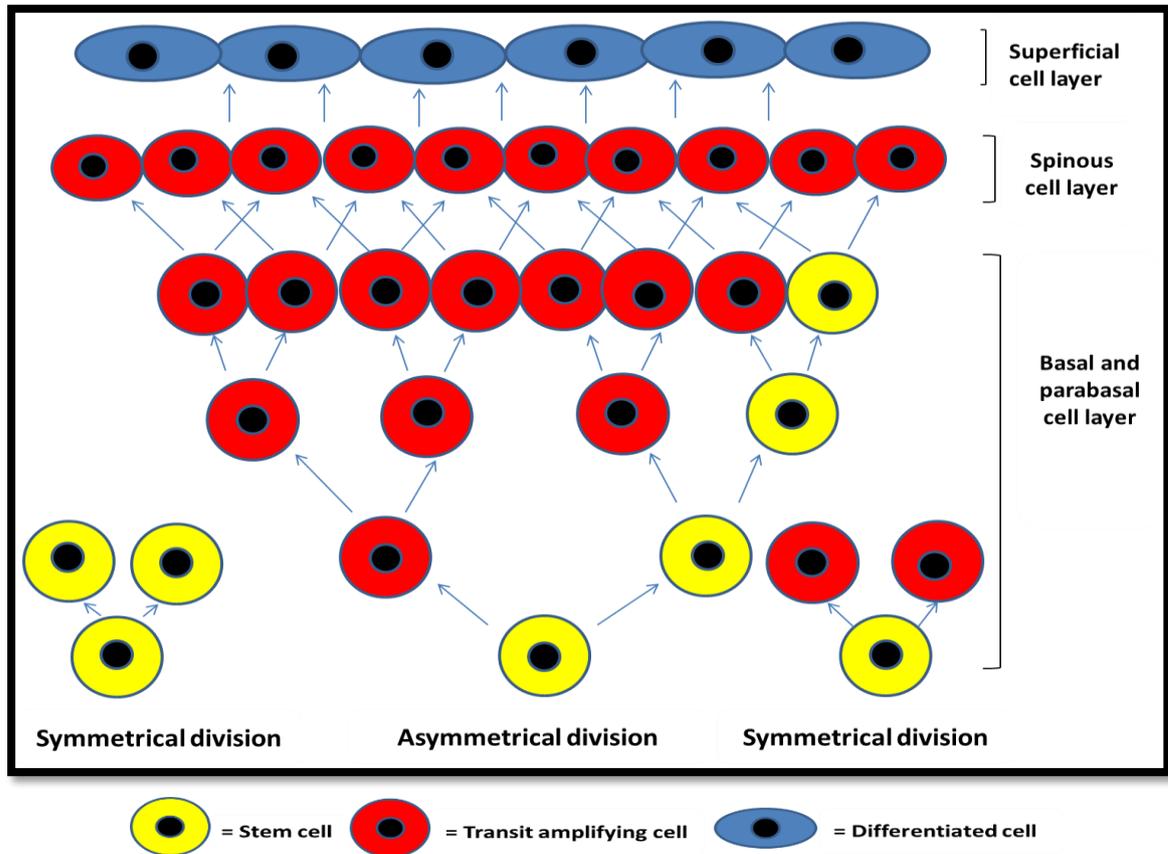


Figure 1.1: Concept of stem cell hierarchy in human oral mucosa.

1.1.3 Types of stem cell

Embryonic stem cells (ESC): are omnipotent cells which are derived from the inner cell mass of the blastocyst. They are considered to be original source of all cells in an organism. Furthermore, they can differentiate to all cell types and generate cells which produce different organs through the differentiation process (Thomson *et al.*, 1998).

Somatic adult stem cells: are immortal cells that reside in various adult tissues. These cells are pluripotent and they continue their biological function throughout the organism lifespan with a more restricted and regulated process of self-renewing. Moreover, there are different types of somatic stem cells that vary in their proliferation and differentiation potential according to the type host tissue. The main

function of these cells are in the renewal of aged tissue and repair of damaged tissues as well as their ability to differentiate into various lineages (Soltysova, Altanerova and Altaner, 2005).

Germinal stem cells: The main function of these specialized cells in adult are production of sperms and eggs (Soltysova, Altanerova and Altaner, 2005).

1.1.4 Normal stem cell niche

The stem cell niche is a specialized microenvironment that forms an indefinite home for one or more stem cells. It is composed of tissue cells and extracellular substrates that regulate the function of these stem cells in maintaining tissue homeostasis and repairing of damaged tissue (Spradling, Drummond-Barbosa and Kai, 2001), as shown in the figure (1.2).

Adult stem cells rely on their niche in supporting and supplying them with the required signals to control their proliferation and differentiation processes. There is a critical balance between these two opposing processes which play a crucial role in prevention of tumourigenesis or depletion of the stem cell pool (Li and Neaves, 2006).

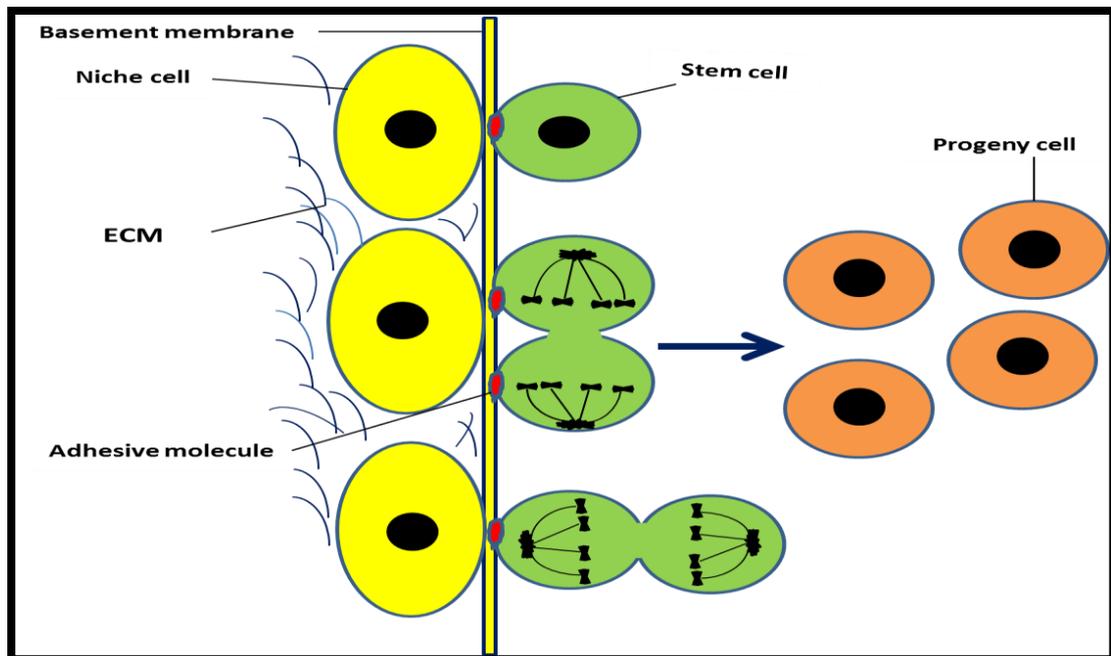


Figure 1.2: Stem cell niche structure, adapted from (Spradling, Drummond-Barbosa and Kai, 2001).

1.2 Cancer stem cells (CSCs)

A small subpopulation of tumour cells have some properties of normal stem cells of the tissue from which the tumour originated from (Maccalli *et al.*, 2014) which include their capability for indefinite self-renewal and differentiation to diverse heterogeneous cell lineages that form the bulk of the tumour (Reya *et al.*, 2001). In addition, cancer stem cells have unique features such as tumourigenicity and have ability to initiate a tumour similar to the original one when they are transplanted to immune deficient animals (Prince and Ailles, 2008). CSCs are considered the most resistant cells in a tumour to conventional anticancer therapy which kill most of the differentiated cancer cells causing shrinkage of tumour except these cells that remain survive and resume growth after cessation of treatment causing subsequent tumour relapse (Vermeulen *et al.*, 2012). Moreover, these cells play a significant role in invasion and metastasis (Campbell and Polyak, 2007).

1.2.1 Cancer stem cells theories

There are two contradictory theories which attempt to explain the initiation and formation of a tumour:

1. The stochastic or clonal theory is the oldest theory and proposes that a tumour arises from mutation of a single normal stem cell giving rise to a neoplastic clone and subsequent a tumour that composed of a homogeneous population of neoplastic cells that all have the same tumourigenic potential (Wang and Dick, 2005).
2. The hierarchical model theory is considered as the most recent theory and proposes that the tumour consists of heterogeneous cell lineages and only a minor subset of cells within it have the ability of tumour initiation and driving the growth of the tumour. These are referred to as cancer stem cells or tumour initiating cells (Wang and Dick, 2005). However, the majority of tumour mass consists of transit amplifying cells which are rapidly proliferating and post mitotic differentiating cells. Both TACs and differentiated cells are derived from cancer stem cells and can not themselves initiate of tumour (Krishnamurthy and Nor, 2012).

1.2.2 Origin of cancer stem cells

The precise origin of CSCs until now remain unclear but there are 3 hypotheses (figure 1.3) that could explain the origin of cancer stem cells:

1. Malignant transformation of normal adult somatic stem cells to cancer stem cells due to accretion of epigenetic and genetic changes (mutations) that lead to loss of their self-regulation of cell proliferation (Smalley and Ashworth, 2003). This hypothesis is supported by the fact that normal stem cells have the longest life-span among all body cells and mutations that lead to transformation of cells is a very slow process requiring a prolonged time, often decades. On the other hand, it is known that cancer stem cells share characteristics of normal stem cells such as self-renewal and differentiation. Whereas, other somatic cells require further mutations to acquire these properties (Islam, Qiao, *et al.*, 2015).
2. Cellular de-differentiation of progenitor cells or fully differentiated mature tumour cells due to mutations that leads to the reacquisition of cancer stem cell properties (Krivtsov *et al.*, 2006). The de-differentiation process is directly linked to epithelial-mesenchymal transition (EMT) (Mani *et al.*, 2008). EMT provides differentiated mature cancer cells with stemness characteristics such as self-renewal and tumorigenicity capabilities that allowing them to generate a tumour at a distant site by metastasis (Qian *et al.*, 2012). Furthermore, invasive cancer cells express both EMT and stemness associated genes (Spaderna *et al.*, 2007).
3. Recently, it has been shown that pluripotent stem cells can be induced from non-pluripotent somatic cells *in vitro* leading to expression of specific genes which subsequently cause reprogramming of these cells via transduction of specific transcriptional factors such as OCT3/4, Sox2, c-Myc and Klf4 (Takahashi and Yamanaka, 2006). The reprogramming process that leads to induced pluripotency and formation of iPS cells similar to the oncogenic transformation process which leads to transformation of somatic stem cells to CSCs (Nishi *et al.*, 2014).

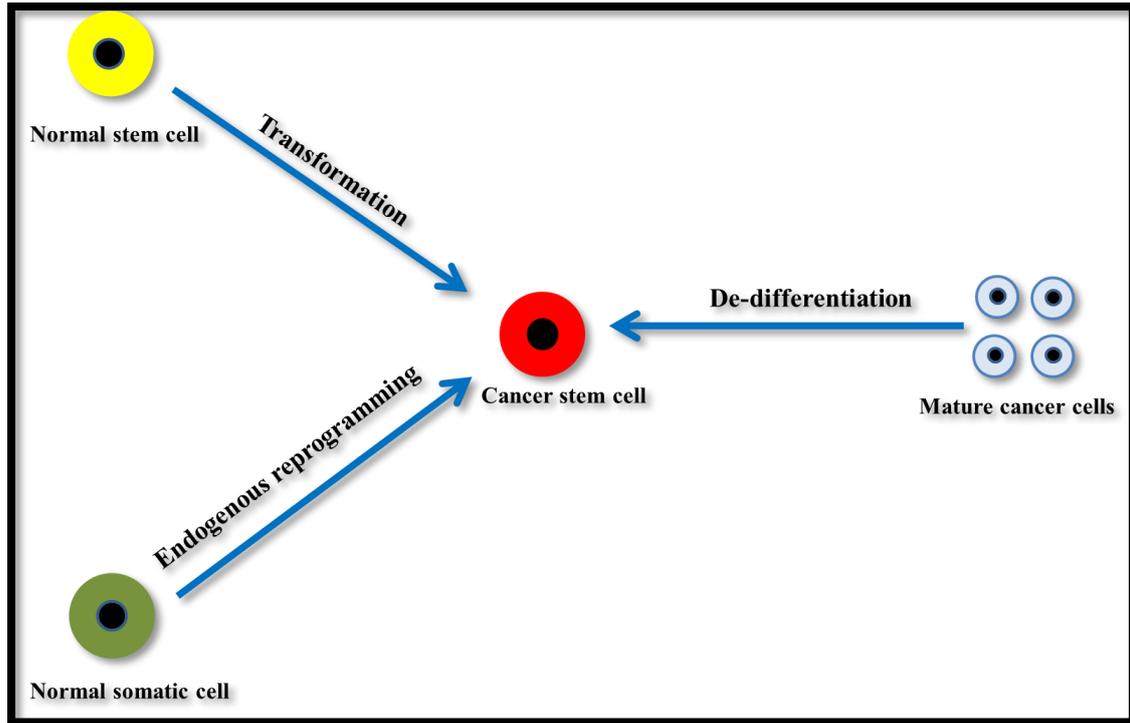


Figure 1.3: Origins of cancer stem cell

1.2.3 Regulation of cancer stem cells

1. Signalling pathways

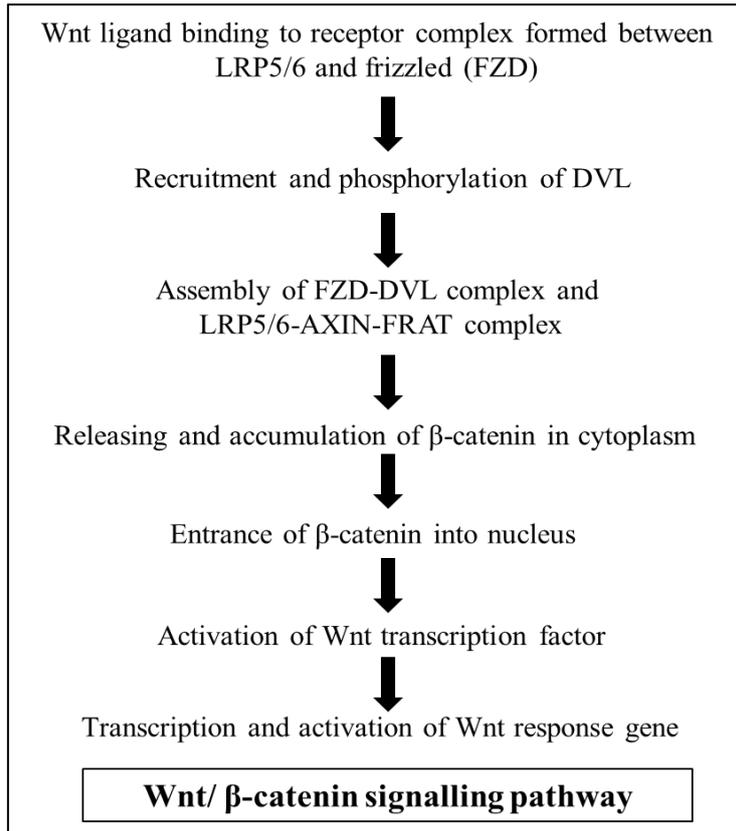
Self-renewal and differentiation of normal stem cells maintain tissue homeostasis via strictly controlled signalling pathways. However, CSCs share normal stem cells pathways but with aberrant activation or dysregulation that leads to tumourigenesis. These signalling pathways are: Wnt / β -catenin, Notch and the hedgehog pathway (Reya *et al.*, 2001) (figure 1.4) that initiate multiple regulatory networks involved in cytokine production in cancer stem cell niche which controls the self-renewal and differentiation of cancer stem cells and subsequent tumour progression (Reya and Clevers, 2005).

The Wnt signalling pathway has a significant role in control of stem cell function through regulation of self-renewal and driving of their symmetrical division (Le Grand *et al.*, 2009). Furthermore, it has been reported that overexpression of this pathway results in de-differentiation of terminally differentiated cells as well as mature cancer cells (Radulescu *et al.*, 2013).

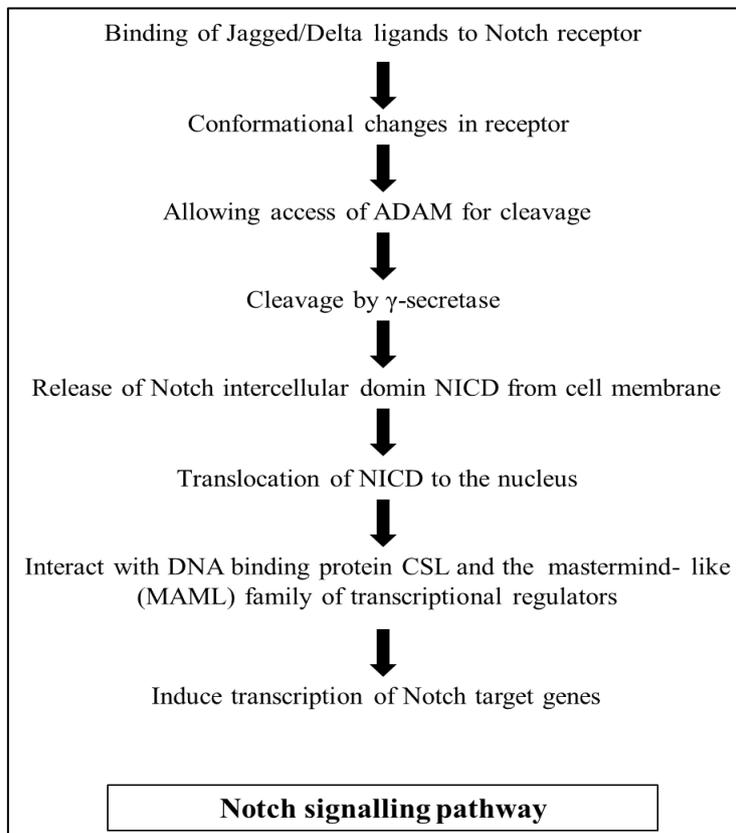
Wnt-target gene activation is mediated by β -catenin. This process involves entrance of β -catenin to the nucleus from the cytoplasm. It then cooperates with the transcription factor TCF/LEF leading to activation of Wnt-target genes such as: *c-Myc*, *cyclin D1* and *c-Jun* (Katoh and Katoh, 2007). On the other hand, it has been identified that aberrant activation of the Wnt signalling pathway has a crucial role in resistance to anticancer therapy resistant. Subsequently, inhibition of β -catenin transcriptional activity is considered an important target in cancer treatment that results in suppressing of tumour growth (Dean, Fojo, and Bates, 2005).

The Notch signalling pathway has been considered as one of key pathways controlling proliferation and differentiation of embryonic and normal adult somatic stem cells (Bray, 2006). However, deregulation of this pathway results in expansion of early progenitor cells and various stem cells (Chiba, 2006). There are 4 types of Notch receptors (Notch 1-4) that have been identified, each one is a non-covalent heterodimer composed of intracellular, transmembrane and extracellular domains. Notch ligands are 2 families: Jagged-like (JLL1/2) and Delta-like (DLL1/3/4). Conformational change in the Notch receptor as a result of binding to its ligand leads to access of a disintegrin and metalloproteinase (ADAM) protein resulting in Notch cleavage followed by another cleavage by γ -secretase which results in release of the Notch intracellular domain (NICD) from the cell membrane which translocates to the nucleus. In the nucleus Notch target gene transcription is induced as a result of an interaction of the NICD with DNA binding proteins (the transcriptional regulators include CSL and the Mastermind-like (MAML) family) (Bray, 2006). HES and HERP gene families are considered the most common primary target genes of Notch signalling pathways (Iso, Kedes and Hamamori, 2003).

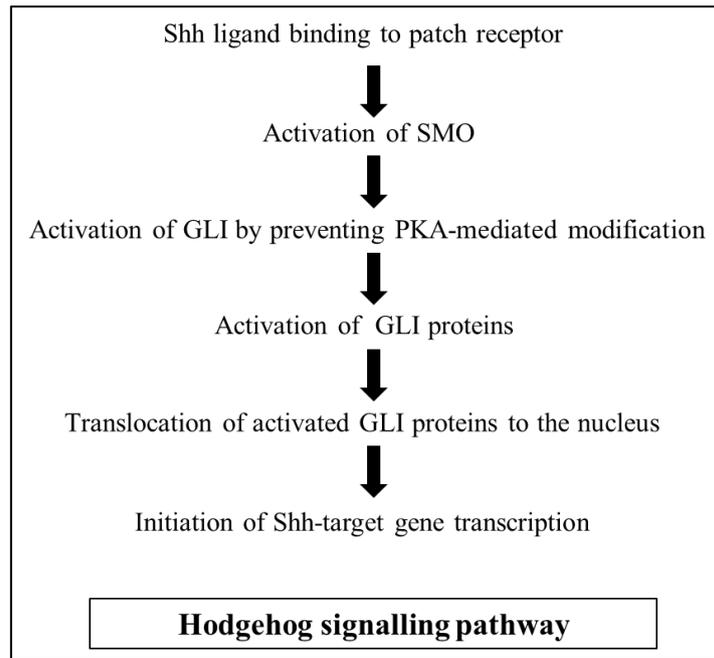
The Hedgehog pathway organises development of organisms through regulation of embryonic stem cells (ESCs), as well as control of the expansion of progenitor and stem cells (Zhao, Li and Guan, 2010). Activation of this pathway is initiated via binding of the Shh ligand to the Patched receptor, which activates the receptor (SMO) leading to induction of a signalling cascade which in turn activates the GLI transcription factors. As a result, Shh target gene expression is initiated which influences cellular proliferation and angiogenesis (Merchant and Matsui, 2010).



-A-



-B-



-C-

Figure 1.4: Diagrams illustrating signalling pathways regulating CSCs (A) Wnt/ β -catenin (B) Notch (C) Hodgehog pathways.

2. miRNA regulation of cancer stem cells

MicroRNAs (miRNAs) are short non-coding RNA molecules which regulate gene expression by targeting the mRNAs either through translational repression or controlled cleavage (Bartel, 2004). MiRNAs are considered as epigenetic factors that participate in tumour progression, anticancer therapy resistance and acquisition of CSCs characteristics (Ji *et al.*, 2009), as well as induction of EMT and subsequent promotion of metastasis (Song *et al.*, 2013). Furthermore, miRNAs play an important role in the control of CSCs functions such as self-renewal, differentiation and tumorigenesis and stimulate expansion of cancer stem cells through regulation of cytokines and chemokines which are produced by cells of the cancer microenvironment (niche) (Yu *et al.*, 2012). Literature suggested that miR-21 and miR-205 are common putative oncogenes that are overexpressed and play important roles in progression of oral cancer (Jiang *et al.*, 2005; Tran *et al.*, 2007; Chang *et al.*, 2008; Carolina, Gomes and Gomez, 2008).

3. Telomerase reactivation

Telomerase is a reverse transcriptase enzyme which is responsible for the addition of terminal repeats to the 3' end of telomeres which are required for cell division

(Allsopp *et al.*, 2003). Shortening of telomeres causes instability of chromosome, fusion and finally senescence (Counter *et al.*, 1992). Therefore, it has been reported that the capacity of cancer stem cells for prolonged self-renewal can be partly attributed to the reactivation of telomerase (Allsopp *et al.*, 2003).

1.2.4 Cancer stem cells and treatment resistance

CSCs play an important role in initiation of cancer when transplanted to immune deficient animals; they are also responsible for recurrence and the relapse of the tumour after chemo or radiotherapy due to their crucial role in resistance to anticancer treatment (Yu *et al.*, 2012). It has been identified that CSCs have an endogenous intrinsic resistance against all conventional anticancer therapies (Bao *et al.*, 2006), i.e. CSCs possess resistance even if the medications have never been applied against a specific tumour previously (Schmidt and Efferth, 2016). As a result, conventional therapies eradicate all cells within a tumour except CSCs (Dallas *et al.*, 2009) (figure 1.5).

CSCs have many mechanisms to protect themselves against destruction and making them resistant to therapeutic chemicals (Takaishi *et al.*, 2009), which include:

1. Aberrations in apoptotic mechanisms

It has been reported that CSCs can avoid the programmed cell death that is normally initiated by conventional anticancer therapy by many mechanisms, such as elevation of the apoptosis induction threshold through increase in the expression of anti-apoptotic proteins, such as the Bcl-2 family of proteins (Madjd *et al.*, 2009). In addition, mutation or inactivation of genes that induce apoptosis as well as cell cycle-regulating genes, for instance p53 gene and its isoforms p63 and p73. Therefore, when the function of p53 gene is lost this promotes EMT via Snail expression and as a consequence increases the therapy resistance (Kim *et al.*, 2011).

2. Elevated expression or high activity of cell membrane transporters (ABC transporters)

CSCs express increased levels of ATP-binding cassette transporter proteins (ABC transporters) which utilize ATP in the translocation of substances such as fat, drugs and metabolic products across the cell membrane. Therefore, they decrease the intracellular levels of the chemotherapeutic agents in CSCs via efflux of drugs outside

the cell membrane (Fletcher *et al.*, 2010). Although the family of ABC transporters involves 49 proteins, only 3 of them are well-characterised as multi-drug resistance regulators in tumours which include ABCB1, ABCC1 and ABCG2 (Vasiliou, Vasiliou and Nebert, 2009).

3. Intracellular detoxification enzymes

The aldehyde dehydrogenase enzyme family are considered the most important detoxification enzymes in CSCs and oxidise intracellular aldehydes to carboxylic acids leading to synthesis of γ -amino butyric acid and retinoic acid that have an important roles in maintaining and differentiating of CSCs (Ma and Allan, 2011). In addition, high activity of ALDH enzymes allow CSCs to metabolize and detoxify various chemotherapeutic agents and their intermediate products, such as cyclophosphamide, ifosfamide, and mafosfamide (Parajuli, Fishel and Hurley, 2014). The detoxification process of chemotherapeutic drugs involves 3 stages. The first stage consists of removing OH and free radical species following transformation to create low toxin metabolites. In the last stage the drug or toxin is pumped outside the cell via membrane channels (Signore, Ricci-Vitiani and De Maria, 2013). It has been reported that high expression of the aldehyde dehydrogenase enzyme ALDH1 in a tumour is a predictor of poor prognosis (Ginestier *et al.*, 2007).

4. Cancer stem cell quiescence

Quiescence is a physiological mechanism by which normal stem cells protect themselves from harmful insults by preventing the exhaustion of their division potential (Maugeri-Saccà, Zeuner and De Maria, 2011). Cancer stem cell quiescence has a significant role in their resistant to treatment based on the fact that the highly proliferating cells within a tumour are targeted mainly by conventional anticancer therapy as a selective mechanism to avoid the non-proliferating normal cells in the body. Whereas, CSCs are usually dormant and proliferate slowly and thus are largely resistant to such therapies (Roesch *et al.*, 2010).

5. Enhanced DNA repair response

Many anticancer therapies induce tumour cell death by causing DNA damage through mechanisms such as: crosslinking of DNA, inhibition of DNA synthesis and inhibition of topoisomerase. If the DNA damage is not repaired, it will be fatal to the

cells (Cheung-Ong, Giaever and Nislow, 2013). However, the highly active DNA repair pathways of CSCs allow them to repair DNA damage effectively. Subsequently, they survive and avoid the effects of therapy (Colak and Medema 2014). The DNA repair process of CSCs include over expression of genes that are involved in DNA damage response systems such as Brca1, Nek1, Hus1, Chek1, Ung, Sfpq, Uhrf1 and Xrcc5 (Zhang *et al.*, 2008). Moreover, activation of cell cycle checkpoints (kinase signalling pathways) which inhibit progression of the cell cycle that allows DNA compensation (Niida and Nakanishi, 2006).

6. Autophagy

Autophagy is a mechanism by which CSCs resist insults from the microenvironment such as starvation, treatment or hypoxia by using energy from alternative sources through activation of catabolic processes that preserve cell viability and metabolic homeostasis. This mechanism involves intracellular degradation and recycling. Proteins and organelles are sequestered in autophagosomes which fuse with lysosomes forming an autolysosome the contents of which are then degraded by lysosomal enzymes resulting in delivery of amino acids and energy (Kroemer, Mariño and Levine, 2010). Recently, it has been reported that CSCs utilise the cytoprotective influences of autophagy in inducing resistance to anticancer therapies in many types of tumour (Ma *et al.*, 2014).

7. Reactive oxygen species (ROS) scavenging

Reactive oxygen species (ROS) are normal metabolism by-products of oxygen and are considered to be a major cause of apoptosis triggered by chemo and radiotherapy (Shi *et al.*, 2012). Radiotherapy leads to extensive production of intracellular ROS that cause toxicity through modification of vital cellular components like DNA, protein and lipid resulting in cell death (Cook *et al.*, 2004). CSCs have high activity of ROS scavengers. Therefore, they are able to keep lower intracellular levels of ROS than non-CSCs (Phillips, McBride and Pajonk, 2006). The ROS scavenging system of CSCs include increased expression of antioxidant enzymes, for instance peroxidase, catalase and superoxide dismutase (Chang *et al.*, 2014).

8. Hypoxia

It has been identified that the usual location of CSCs in a tumour is near to hypoxic zones (Simon and Keith, 2008). Therefore, hypoxia is thought to participate in maintaining CSCs self-renewal as well as inducing of EMT (Das *et al.*, 2008). At low levels of oxygen, the hypoxia response is initiated through activation of hypoxia – inducible factor 1 alpha (HIF1 α), which is then translocated into the nucleus and dimerizes with HIF1 β leading to activation of transcription of hypoxia response genes (Semenza, 2004). More than 60 response genes have been identified which activate survival signalling pathways for proliferation as well as promotion of angiogenesis to provide oxygen (Michiels, 2004). The role of hypoxia in therapy resistance is attributed to activation of stem related signalling pathways and promotion of quiescence. Therefore, expression of both Wnt, Notch and Hedgehog signalling pathways and stemness markers such as c-MET, NANOG and SOX2 are induced via activation of HIF1 α (Majmundar, Wong and Simon, 2010). However, CSC quiescence is induced as a result of unfavourable conditions for cell proliferation such as limitations of nutrients and reduction of oxygen that occurs during hypoxia (Almog, 2010).

9. Epithelial mesenchymal transition (EMT).

EMT is a process of changing of the polarity and epithelial phenotype of epithelial tumour cells to a mesenchymal, fibroblast-like phenotype, which results in the loss of epithelial integrity and increased of migration and subsequent local invasion and metastasis (Thiery, 2002). It has been reported that anticancer resistance is enhanced in CSCs undergoing EMT at the same time in response to specific pathways such as the TGF- β pathway (Sarrio *et al.*, 2012).

10. MicroRNA deregulation

It has been reported that deregulation of specific microRNAs have a role in promoting the CSCs phenotype and acquiring resistance to anticancer therapy as a consequence (MacDonagh *et al.*, 2015).

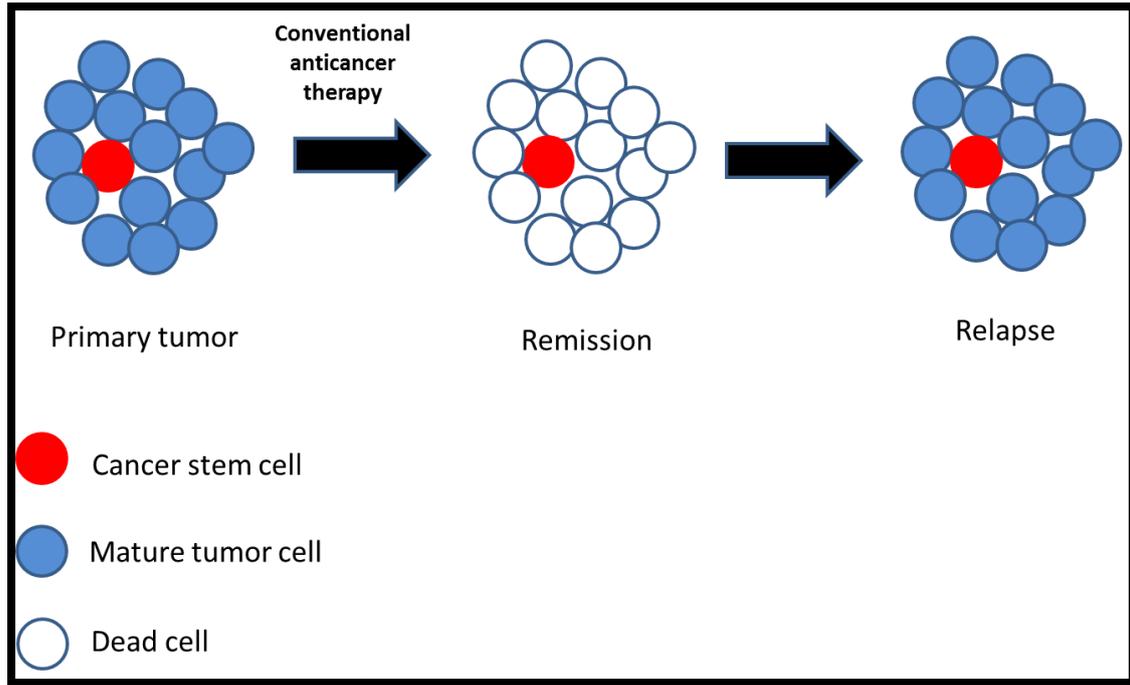


Figure 1.5: Effects of conventional anticancer therapy on tumour progression.

1.2.5 Cancer therapy targets

1. Signalling pathways inhibition

Inhibiting CSC self-renewal and proliferation signalling pathways, such as Wnt, Notch and Hedgehog pathways are considered to be one way to target CSCs (Kim, Jeon and Kim, 2014). The Wnt pathway can be targeted by blocking the transcription of Wnt targeting genes using biological inhibitors or small molecule antagonists (Fujii *et al.*, 2007). These include small interfering RNA (siRNA), monoclonal antibodies, non-steroidal anti-inflammatory drugs such as Sulindac which target β -catenin (Boon *et al.*, 2004), and small molecule antagonists like FJ9 that target Dvl (Fujii *et al.*, 2007). Moreover, Notch signalling can be directly targeted using γ -secretase inhibitors which prevent enzymatic cleavage of Notch and NICD release (Mizugaki *et al.*, 2012; Leon *et al.*, 2016). The Hedgehog pathway can also be targeted by inhibiting either the SMO receptor or inhibition of the Gli transcription factor (glioma associated oncogene homolog) (Ng and Curran, 2013). Although blocking and down-regulating signalling pathways is considered an effective approach for CSCs targeting, they may have negative impacts on normal stem cells. Therefore, these approaches should be modified to improve their specificity (Han *et al.*, 2013).

2. CSC induced apoptosis

Another therapeutic approach would be to shift the apoptotic balance towards a more pro-apoptotic state by inhibiting anti-apoptotic proteins (Colak *et al.*, 2014). For instance, induction of apoptosis in CSCs in some tumours due to over-expression of tumour necrosis factor-related apoptosis (TRAIL) which is a pro-apoptotic protein and inhibition of NK-kB which is an anti-apoptotic transcription factor (García *et al.*, 2012).

3. Inhibition of ABC transporter

Targeting ATP-binding cassette transporters and drugs efflux pumps in CSCs would subsequently reduce the clearance of chemotherapeutic drugs (Papagerakis *et al.*, 2014). Verapamil is an example of general inhibitor for ABC transporters that increased the chemo-sensitivity of CSCs (Loebinger *et al.*, 2008).

4. Targeting of CSC surface markers

Although surface markers have been widely used in detection and identification of CSCs, they also provide a strategy to target these cells (Leon *et al.*, 2016). Therefore, antibody based therapies which include immunotoxins that are directed to CSCs markers specifically have been proposed (Swaminathan *et al.*, 2013).

5. Targeting of CSC quiescence

This was performed by enhancing proliferation in order that they respond to conventional therapy (Colak *et al.*, 2014). Sensitivity to anticancer therapies could be restored by inducing quiescent CSCs to enter the cell cycle. Cytokines such as granulocyte-colony stimulating factor (G-CSF) and interferon- α (IFN α) play a key role in the promotion of dormant CSCs cycling (Essers and Trumpp, 2010).

6. Inactivation of DNA damage repair mechanisms

It has been investigated that a potential therapy was obtained using agents that interfere or block the mechanisms of DNA repair such as Poly (ADP-ribose) polymerase (PARP) and DNA-dependent protein kinase (Veuger *et al.*, 2003). In addition, inhibition of checkpoint kinases (Chk1 and Chk2) by specific inhibitors enhanced the blocking of DNA repair mechanisms (Reinhardt and Yaffe, 2009).

7. Targeting of the CSC niche (microenvironment)

CSCs depend on their interaction with the tumour niche for their survival, self-renewal, differentiation and tumourigenesis. Therefore, blocking stromal signalling systems in the CSCs niche (Ma *et al.*, 2013), as well as disruption and interfering with the prevasculature niche may represent other possible cancer therapy routes (Krishnamurthy *et al.*, 2010).

8. MicroRNA expression manipulation

It has been reported in several tumours that miRNAs act as tumour suppresser genes which target and down-regulate many oncogenes such as miRNA-34a that target Notch-1, Notch-2, c-Met and CDK6 (Guessous *et al.*, 2010). Thus, manipulation of miRNA may represent another strategy to target CSCs.

9. Targeting the hypoxic conditions of the tumour microenvironment

Inhibition and inactivation of hypoxia inducible factor 1- α (HIF1 α) is considered a key factor in increasing oxygen levels and targeting hypoxia in the tumour microenvironment. Furthermore, it has been shown that LY294002 and rapamycin are the most common HIF1 α inhibitors (Zhong *et al.*, 2000).

10. Inhibiting the activity of detoxification enzymes

Aldehyde dehydrogenase enzymes (ALDH) can be blocked by specific inhibitors such as diethylaminobenzaldehyde (DEAB) and/or all-trans retinoic acid (ATRA) which lead to increased sensitization of CSCs to anticancer therapies (Croker and Allan, 2012).

11. Induction of CSCs differentiation

This approach of treatment involves forcing CSCs to terminal differentiate and therefore lose their ability to self-renewal. Anticancer differentiation treatment includes 2 group of drugs: First, retinoic acid (vitamin A and retinoid), and secondly drugs that are directed against tumour epigenetic changes such as all-trans retinoic acid (ATRA), suberoylanilide hydroxamic acid (SAHA) and Histone deacetylase (HDAC) inhibitors (Massard, Deutsch and Soria, 2006).

However, several therapeutic approaches have been suggested to target CSCs and most of them currently are the research phase. Targeting of self-renewal and proliferation signalling pathways of CSCs are entering clinical trial phases followed by targeting of stromal signalling pathways in CSCs microenvironment to a lesser extent (Liu and Wicha, 2010; Takebe *et al.*, 2015).

1.3 Oral cancer

1.3.1 Prevalence

Oral cancer is considered to be the eighth most common around the world (Amit *et al.*, 2013). Oral squamous cell carcinoma represents about 90% of oral cancers and there are about 300,000 new cases diagnosed worldwide every year (Ferlay *et al.*, 2015). The tongue is considered as the most common site for oral cancer followed by the floor of mouth, while other intra-oral sites such as gingiva, buccal mucosa, labial mucosa and hard palate are less common sites (Boffetta *et al.*, 2008). Despite advances in diagnosis and treatment methods of oral cancer over the last few decades, there has been no significant improvement in patient prognosis. Furthermore, the survival rate of patients with oral cancer remains very low with less than 50% survival after 5 years because of late detection and the high risk of recurrence and development of a second primary tumour. This survival rate has remained largely unchanged over the last 50 years (Tanaka *et al.*, 2011).

1.3.2 Risk factors

There are many risk factors which play a significant role in oral tumorigenesis either independently or synergistically (Tanaka *et al.*, 2011), which include:

1. Tobacco

Tobacco consumption in all its forms whether smoked, chewed or taken as snuff is considered an important traditional oral carcinogen in adults (Llewellyn, Johnson and Warnakulasuriya, 2001). It has been reported that 1/4 of oral cancer cases due to cigarette smoking (Hashibe *et al.*, 2007). Furthermore, it has been identified that cigarette smoke contains more than 60 carcinogens. Tobacco-specific nitrosamines,

polycyclic aromatic hydrocarbons and aromatic amines are the most important carcinogens present in cigarette smoke and are considered causal factors linked with oral cancer (Hecht, 2003). The association between oral cancer and cigarette smoking is dose dependent, i.e. the risk of developing oral cancer rises proportionally with the number of cigarettes smoked daily and duration of smoking (Hashibe *et al.*, 2007).

2. Alcohol

Heavy alcohol drinking is considered the main cause of about 7-19% of oral cancer cases (Hashibe *et al.*, 2007). It has been reported that the incidence of oral cancer among heavy drinkers is 2-3 fold higher than non-drinkers (Seitz and Stickel, 2007). Alcohol is metabolised by alcohol dehydrogenase (the major alcohol metabolising enzyme) which oxidises ethanol to acetaldehyde which is considered an oral carcinogen that accumulates in the oral mucosa of heavy drinkers with consequent production of DNA adducts (Baan *et al.*, 2007; Seitz and Stickel, 2007). In addition, alcohol exerts other carcinogenic effects through several suggested mechanisms. First, alcohol may act as solvent to facilitate penetration of other carcinogens through cellular membranes of targeted tissues. Second, ethanol may activate carcinogenic substances by stimulating liver metabolism. Third, it may alter the intracellular metabolism of epithelial cells in the target tissue (Llewellyn, Johnson and Warnakulasuriya, 2001).

3. Betal quid

Chewing of betal quid has damaging effects on the oral mucosa either through direct initiation of tumour inducing mutations or by rendering the mucosa susceptible to environmental poisons (Merchant *et al.*, 2000).

4. Viral infection

There are two types of viruses have been implicated in oral carcinogenesis which are Human papilloma virus (HPV) and Epstein-Barr virus (EBV) (Llewellyn, Johnson and Warnakulasuriya, 2001).

5. Immune deficiency

As a result of the increased incidence of oral cancer in immune deficient people. It is considered a predisposing factor for oral cancer in young individuals. For instance, human immune deficiency virus infection (HIV infection), Wiskott-Aldrich syndrome, Bloom syndrome, Plummer Vinson syndrome and immunosuppression regimes after organ transplantation (Scully *et al.*, 1991; Streilein, 1991; Varga and Tyldesley, 1991).

6. Familial and genetic factors

It has been increasingly evident that oral cancer development has a familial and genetic predisposition especially in young people with no other associated risk factors. There is a significant increase in fragility of chromosomes after exposure to mutagens in some individuals compared to old people. The chromosomal fragility might result in genetic abnormalities (e.g. alterations of DNA repair genes such as XRCC1 and XRCC3 repair genes) in tobacco smoking young adults which may contribute to the development of head and neck oral cancer (Kostrzewska-Poczekaj *et al.*, 2013).

7. Diet

Several epidemiological studies have been suggested that the diet play a role in the development of oral cancer. High levels of vitamins and antioxidants found in vegetable and fruits may have a protective role against oral cancer (Negri *et al.*, 2000). On the contrary, food riche with animal fat, animal protein, cholesterol and saturated fatty acids may be increasing the risk of oral cancer (Bravi *et al.*, 2013).

1.3.3 Tumorigenic process

Oral cancer arises as a consequence of many molecular events which may occur due to the combined effects of exposure to environmental carcinogens and an individual's genetic predisposition (Califano *et al.*, 1996). These events include damage of individual genes and genetic material due to chronic exposure to carcinogens. Accumulation of genetic alterations such as mutations, oncogene amplification and tumour suppressor genes inactivation will result in the formation of a premalignant

lesion and subsequent carcinoma. Therefore, the tumour cells will acquire autonomous self-sufficient growth and elude the inhibitory growth signals leading to immortalization due to telomere lengthening and subsequently result in uncontrolled progression of tumour. In addition, endothelial cells are stimulated to create new blood vessels (angiogenesis) which have an essential role in progression of solid tumour (Hanahan and Weinberg, 2000).

1. Acquisition of self-sufficient growth

Proliferation of normal cells requires exogenous signals provided by growth factors. These factors usually interact with the extracellular matrix (ECM) and cytokines (Hanahan and Weinberg, 2000). These signals are transduced from cell surface-receptors leading to activation of many intracellular pathways and subsequent stimulation of cell proliferation (Todd *et al.*, 1991). In oral carcinogenesis, there is a dysregulation of growth factor signalling due to increased levels of receptor and/or growth factors ligands, which enhances autocrine stimulation in the absence of exogenous factors and results in tumour progression (Grandis and Tweardy, 1993). The growth factors include: epidermal growth factor (EGF), hepatocyte growth factor (HGF) and activating protein-1 (AP-1) (Choi and Myers, 2008).

2. Abnormalities in growth-inhibitory signals

During oral carcinogenesis, the expression of cell cycle inhibitory proteins encoded by tumour suppresser genes is lost. In normal cells, there is tight regulation of the growth inhibitory signals by the interaction of cyclin-dependent kinases (CDK), cyclins, and the product of the retinoblastoma (Rb) gene. In addition, other inhibitors of cell-cycle progression are proteins encoded by the tumour suppressor genes p16, p15, p21, and p53 (Choi and Myers, 2008).

3. Evasion of Apoptosis

Apoptosis is a tightly regulated physiologic cellular mechanism for programmed cell death to eradicate the altered or senescent cells which become harmful or useless and is considered to have crucial role in cell homeostasis (Williams, 1991) .

In normal cells, apoptosis is controlled by the Bcl-2 family of regulatory proteins which consist of about 15 proteins and are divided into anti-apoptotic (Bcl-2, Bcl-XL) or pro-apoptotic (Bax, Bak) proteins. In carcinogenesis the expression of Bcl-2 family

proteins is altered and leads to a change in the pro-apoptotic/ anti-apoptotic protein ratio. As a consequence, inhibition of apoptosis occurs resulting in promotion of tumour growth (Kroemer, 1997). Moreover, Popović *et al.*, (1996) reported an enhance expression of anti-apoptotic proteins Bcl-2 and Bcl-XL in oral squamous cell carcinoma and oral dysplastic lesions.

4. Immortalization

Normal cells have a finite replication capacity and after certain number of cell divisions, they terminate proliferation and become senescent. On the contrary, the capacity of replication of tumour cells is unlimited and they become immortalized due to lengthening of their telomeres through expression of telomerase (Stewart and Weinberg, 2000). Several oral cancer studies have identified the overexpression of human telomerase reverse transcriptase (the protein catalytic subunit of telomerase) is an early event in oral carcinogenesis (Lee *et al.*, 2001; Chen *et al.*, 2007).

5. Angiogenesis

Growth of tumours beyond a certain size requires the formation of new blood vessels from pre-existing ones, which is achieved by shifting the balance of pro-angiogenic / anti-angiogenic factors (Folkman, 1990). The factors that regulate the angiogenic process are divided into pro-angiogenic signals such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), acidic and basic fibroblast growth factors (FGF1 and FGF2), and IL-8 and the inhibitory signals which are the interferons, proteolytic fragments such as angiostatin and endostatin, and thrombospondin-1 (Choi and Myers, 2008). In addition, Tanigaki *et al.*, (2004) found a significant correlation between overexpression of pro-angiogenic VEGF-C with local recurrence and distant metastasis in oral squamous cell carcinoma.

1.3.4 Invasion and metastasis

Oral cancer is characterised by invasion of adjacent tissues as well as dissemination to cervical lymph nodes and distant sites (figure 1.6). Invasion and metastasis are considered a complex process involving many steps such as adhesion of cells, re-organisation of the cell cytoskeleton, migration of cells, basement membrane dissolution, intravasation, survival of cells in the blood stream, extravasation and

growth at the distant site accompanied by neo-angiogenesis (Chambers, Groom and MacDonald, 2002). In the first step of invasion, the motility and invasiveness of tumour cells will be increased, and it has been assumed that these events may be related to epithelial mesenchymal transition (EMT). Furthermore, polarity loss and motility acquisition require loss of cell to cell adhesion, cytoskeletal rearrangements and organelle redistribution including changes in gene expression profiles of tumour cells (Thiery, 2002). Alterations in expression of E-cadherin and integrins play a significant role in EMT and tumour invasion. E-cadherin is cell adhesion molecule that maintains epithelial cell integrity through mediating cell to cell adhesions between normal mucosal cells. Therefore, changes in E-cadherin expression or activity leads to an alteration in the shape of cells and an increase their motility and invasiveness. Integrins are cell-surface receptors that binds the epithelial cells to the extracellular matrix (ECM). Alterations in integrin expression will alter cell attachment making cells more motile and invasive. In addition, local invasion requires dissolution and disintegration of basement membrane and modification of the ECM mediated by proteolytic enzymes such as matrix metalloproteinases (Choi and Myers, 2008).

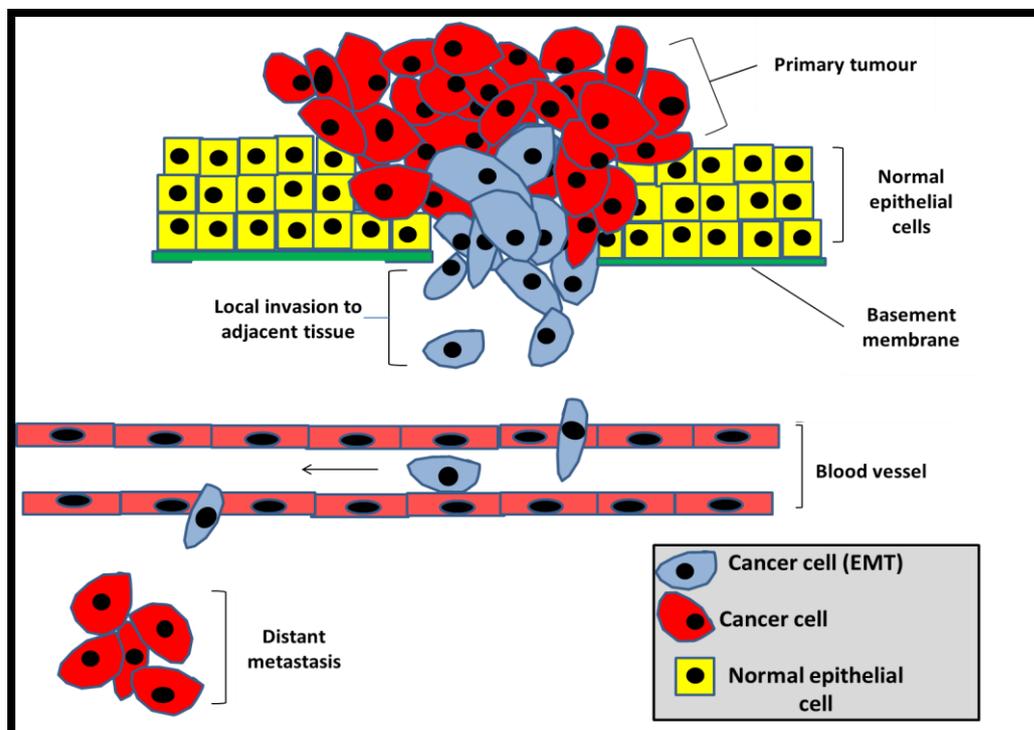


Figure 1.6: Cancer invasion to adjacent tissue and distant metastasis.

1.3.5 Tumour microenvironment

The tumour microenvironment is a complex system resulting from a crosstalk between various cell types and it plays a crucial role in cancer progression because it is considered as an essential regulator of carcinogenesis and angiogenesis. It is composed of invasive tumour cells and stromal elements which include fibroblasts of different phenotypes, ECM and inflammatory and immune cells as well as blood vessels, lymph vessels and nerves (Albini and Sporn, 2007) (figure 1.7).

The most important stromal element is cancer associated fibroblast (CAF) which has a critical role in tumorigenesis and angiogenesis by providing a communication network. CAFs secrete chemokines such as CXCL12 which induce angiogenesis and also they secrete growth factors that induce altered ECM consequently providing further oncogenic signals that stimulate proliferation and invasion of cancer cells (Kalluri and Zeisberg, 2006). Myofibroblasts are another important element which are activated when the integrity of tissue is compromised and invasion begins. They may increase growth and expansion of tumour via secretion of numerous growth factors, chemokines, cytokines, and ECM components (Shao, 2006). Other cellular components of tumour microenvironment which take part in tumour growth include: endothelial cells, adipocytes, smooth muscle cells, neutrophils, mast cells, lymphocytes, macrophages and dendritic cells (Albini and Sporn, 2007).

There is also a variety of proteins secreted by tumour cells into the microenvironment which include growth factors and ECM degrading proteinases. These secreted proteins are implicated in cell adhesion, motility, cell-cell communication and invasion (Albini and Sporn, 2007).

The ECM of microenvironment is remodelled and modified extensively by proteases particularly matrix metalloproteinases (MMPs) secreted from tumour and non-tumour cells which result in changes in cell to cell and cell to ECM interactions and the generation of new signals from the cell surface (Nakajima et al., 1987). Furthermore, the non-ECM proteins such as cytokines, growth factors and growth factors receptors are also targeted by MMPs. Growth factors can be stored in the ECM and released and activated by MMPs (Fingleton et al, 2001).

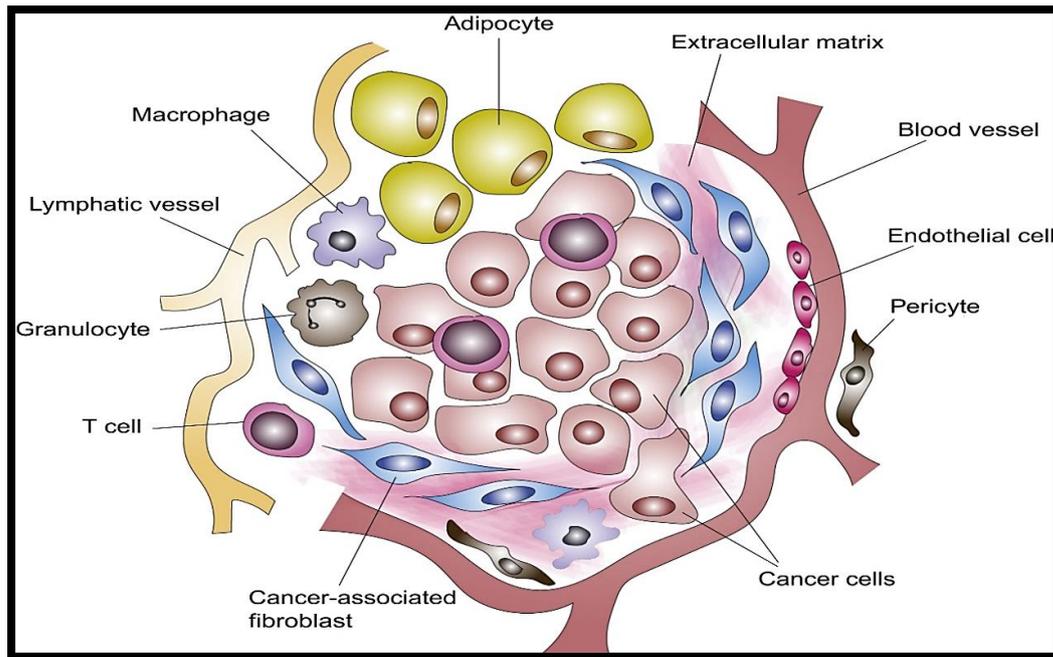


Figure 1.7: Tumour microenvironment adapted from (Prajapati and Lambert, 2016), permission obtained to reuse here.

1.3.5.1 Fibroblast phenotypes in the tumour microenvironment

Fibroblasts are non-vascular non-inflammatory mesenchymal cells present in the fibrillar matrix of connective tissue that they secrete. The normal functions of these cells is maintenance of homeostasis of both the adjacent epithelia through secretion of growth factors and of the extracellular matrix ECM through synthesis of collagen I, III, V and fibronectin as well as matrix metalloproteinases (the degrading enzymes of ECM) (Tomasek *et al.*, 2002). Although fibroblasts usually exist in a dormant state in normal conditions, they are triggered and transiently activated during wound healing and invade the wound site and acquire a myofibroblast phenotype with enhanced expression of alpha-smooth muscle actin (α -SMA) stress fibres (figure 1.8). Furthermore, wound contraction and closing are attributed to the effects of myofibroblasts laying down of ECM and their contractile properties. Subsequent scar formation can be initiated by rearrangement of new ECM by MMPs secreted from the activated fibroblast (Darby *et al.*, 2014). As soon as the wound is repaired, a significant reduction the number of activated fibroblasts occurs accompanied by a restoration of fibroblast phenotype. To date, it is not known if the activated fibroblasts are cleared by apoptosis and replaced from adjacent normal tissue with resting

fibroblasts or whether activated fibroblasts return to their normal quiescent phenotype that they had before activation (Tomasek *et al.*, 2002). On the other hand, cancer associated fibroblasts (CAFs) are permanently activated in the tumour microenvironment, have stable phenotype even in absence of the activation stimuli, and are not eliminated by apoptosis nor revert to their original normal phenotype (Li, Fan and Houghton, 2007) (figure 1.8).

1.3.5.2 Activation of normal fibroblasts into CAFs

Transforming growth factor β (TGF- β) is considered as the key pathway that induces activation of both cancer associated fibroblasts (CAFs) and physiologically activated fibroblasts. Although, there are other growth factors that mediate activation of quiescent fibroblasts such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor 2 (FGF2), chemokines like monocyte chemoattractant protein-1 (MCP1) and ECM-degrading proteases (Kalluri and Zeisberg, 2006). It has been reported that tumour cells directly trigger the activation and transition of normal fibroblasts to CAFs (Vicent *et al.*, 2012). However, some studies suggested that the interaction of normal fibroblasts with ECM has an important effect on their activation, for instance TGF- β 1 induced differentiation of myofibroblast from oral and dermal fibroblasts is inhibited by blocking of the α 5 β 1 integrin (Lygoe *et al.*, 2007). On the other hand, already activated CAFs play a significant role in further recruitment of resting quiescent residential fibroblast which acquire a CAF phenotype through homotypic interactions (Vicent *et al.*, 2012). In addition, Clayton and his colleagues (1998) revealed that leukocytes activate normal fibroblast directly by cell to cell communication via adhesion molecules like vascular cell adhesion molecule-1 (VCAM-1) and/or intercellular adhesion molecule-1 (ICAM-1).

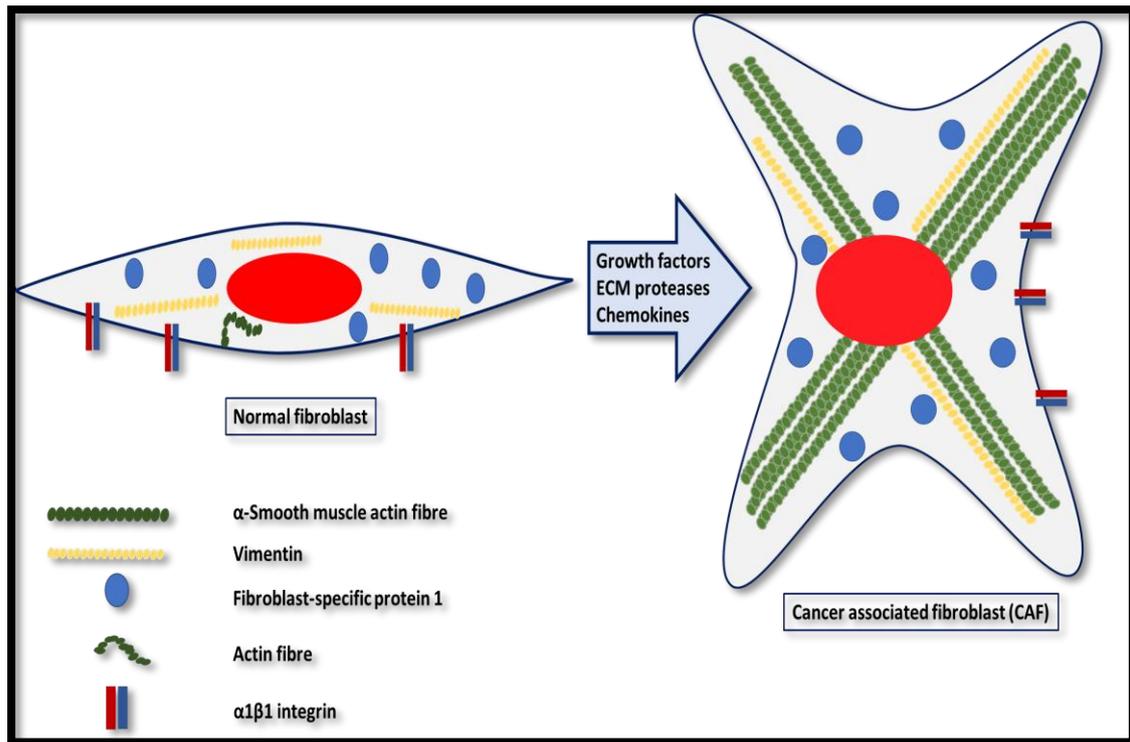


Figure 1.8: Schematic representation illustrating the morphological and proprieties differences between normal fibroblast and cancer associated fibroblast. Adapted from (Kalluri and Zeisberg, 2006).

1.3.5.3 Transforming growth factor (TGF- β)

TGF- β is a pleiotropic growth factor that displays dual activities, it has a tumour suppression effect in normal cells, whilst acting as tumour promoter in cancer cells through induction of an invasive phenotype (Massagué, 2008). In addition, TGF- β plays significant roles in metastasis and angiogenesis (Costanza *et al.*, 2017) as well as mediation of epithelial- mesenchymal transition (EMT) (Yu *et al.*, 2014) and activation of cancer associated fibroblasts (Kalluri and Zeisberg, 2006). Although, TGF- β possess four variants, TGF- β 1 has received the most experimental attention due to its production by a variety of cells such as keratinocytes, macrophages, fibroblasts, and platelets (Lee and Eun, 1999; Mani *et al.*, 2002; Eppley, Woodell and Higgins, 2004). Moreover, it has been reported that TGF- β 1 has other important activities such being chemotactic to inflammatory cells, fibroblasts and keratinocytes, promotion of inflammatory cell recruitment (Moses, Yang and Pietenpol, 1990) and hyperplastic scaring regulation (Russell *et al.*, 1988).

1.3.5.4 Cancer associated fibroblasts (CAF)

CAFs represent the activated phenotype of fibroblasts within the tumour microenvironment and are characterised by their high expression of α -SMA. They form the most common cells within stroma in most tumour types (Kalluri and Zeisberg, 2006). CAFs enhance tumour progression by triggering proliferation of tumour cells through secretion of different growth factors, cytokines and hormones. For instance, epithelial mitogens like hepatocyte growth factor (HGF), fibroblast growth factor (FGF), Wnt family proteins and epidermal growth factor (EGF) as well as cytokines such as, interleukin-6 (IL-6) and stromal derived factor (SDF)-1 α (CXCL12). CAF expression of all these factors is increased in various tumour types (Pietras and Östman, 2010). In addition, CAFs secrete many chemokines such as monocyte chemoattractant protein 1 (MCP1) and cytokines as well as interleukins such as (IL-1) to initiate an inflammatory response in tumours (Strieter et al., 1989; Kalluri and Zeisberg, 2006). Moreover, CAFs enhance remodelling of ECM through secretion of ECM proteins such as collagen I, fibronectin and tenascin C as well as ECM degrading proteases such as MMP2, MMP3 and MMP9 (Kalluri and Zeisberg, 2006). CAFs have an important role in promotion of angiogenesis through secretion of factors which stimulate endothelial cells and pericytes (Hanahan and Coussens, 2012). For instance, endothelial progenitor cell recruitment is mediated by CXCL12 secreted from CAFs (Orimo *et al.*, 2005). It has been reported that CAFs are crucial in suppression of immune cells recruitment and modulation of the immune response. The immunosuppressive activity of fibroblast activation protein (FAP) expressing CAFs was attributed to their excessive secretion of CXCL12 that results in elimination of programmed cell death ligand 1 (PD-L1) and anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) which is an immunological checkpoint antagonists responsible for promotion of T cell function (Feig *et al.*, 2013).

1.3.5.5 Origin of CAFs in tumour microenvironment

CAFs in tumour stroma vary in their phenotypes, morphology and biological properties. This heterogeneity could be explained by the variety of their origins (Rønnov-Jessen *et al.*, 1995). CAFs could be derived by activation of local tissue resident fibroblasts or could arise from epithelial cancer cells that undergo epithelial-

mesenchymal transition (EMT). Moreover, CAFs might originate from endothelial cells and pericytes through endothelial-mesenchymal transition (EndoMT) the process by which endothelial cells convert to fibroblast-like cells (Orimo and Weinberg, 2007). In addition, recruitment and activation of cell populations from distant tissues such as bone marrow progenitor cells and circulating fibrocytes is considered another possible source of CAFs within the tumour microenvironment (Ishii *et al.*, 2003).

1.3.5.6 CAF markers

Previously α -SMA, fibroblast activated protein (FAP), platelet derived growth factor receptor- α or β (PDGFR- α or β), neuron glial antigen (NG2), podoplanin, and thy-1 (CD90) were considered as specific markers for identification of CAFs (Sugimoto *et al.*, 2006, Polanska and Orimo, 2013). Currently, it has been widely identified that there is no specific marker expressed by CAFs that is not expressed by other type of cells, for example: vascular smooth muscle cells expressed high levels of α -SMA. Neuron glial 2 (NG2) and platelet derived growth factor receptor- β (PDGFR- β) are well known to be found in normal pericytes, while podoplanin is expressed by lymphatic endothelial cells (Augsten, 2014). This fact could be attributed to several causes which are: the variety of origins of CAFs, tumour microenvironment heterogeneity and the resemblance of CAFs to normal host fibroblasts (Bussard *et al.*, 2016). Therefore, currently the most reliable methods to identify CAFs are dependent on a combination of both marker expression and morphological appearance (Ishii *et al.*, 2016).

1.3.5.7 Heterogeneity of CAFs

It has been reported that CAFs do not have a unique phenotype, genotype and secretory potential within tumour stroma, even in stroma of a specific tumour (Lewis *et al.*, 2004; Rosenthal *et al.*, 2004; Sugimoto *et al.*, 2006; Tchou *et al.*, 2012). Recently, it has been found that CAF diversity and the cooperation between various cell subpopulations play a pivotal role in tumorigenesis (Kiskowski *et al.*, 2011). In oral squamous cell carcinoma (OSCC), two different subtypes of CAF have been identified. CAF-N which is close to normal fibroblasts in their secretory profile and

gene expression and CAF-D which has divergent gene expression with increased levels of TGF- β 1 secretion. A high percentage of cells are CAF-N and exhibit intrinsic motility and their migration potential is increased in response to TGF- β 1. However, CAF-D involves only a few motile cells and their motility capacity is largely unaltered in response to TGF- β 1. The authors assumed that the various detected phenotypes (CAF-N and CAF-D) are not actually distinct phenotypes, but they represent different stages of CAF progression in OSCC, where CAF-N exist in early stage which later on change to CAF-D. Moreover, this changing process is regulated and controlled by the levels of TGF- β (Costea *et al.*, 2013).

1.3.5.8 Senescent fibroblasts

Cellular senescence can be defined as a state of permanent growth arrest of cells due to loss of their ability to proliferate. However, cells maintain their viability as well as metabolic and transcription activities. Senescent cells differ from quiescent cells by the elevated levels of p^{INK4A} (Hayflick, 1965). The main cause of cellular senescence is irreparable DNA damage. This damage may be induced by telomeres shortening due to excessive replication or mutations (Crabbe *et al.*, 2004) as well as oxidative stress that result from mitochondrial dysfunction, oncogene overexpression and anticancer therapies (Di Micco *et al.*, 2006). The p53 or p16/Rb pathway stimulate the G1-S checkpoint to arrest the growth of cells and initiate cellular senescence (Coppé *et al.*, 2008; Demehri, Turkoz and Kopan, 2009; Pazolli *et al.*, 2009). Evidence suggests that fibroblast activation and senescence represents various stages of the same pathway (Hassona *et al.*, 2013). Support of this theory comes from the fact that activated fibroblasts (TGF- β treated) possess a gene expression profile which significantly overlaps with senescent fibroblasts (radiation-treated fibroblasts). Also, when oral fibroblasts treated with TGF- β result are activated this is followed by senescence. An α -SMA positive phenotype is expressed by activated and senescent CAFs and finally both activated and senescent CAFs have the same tumour promoting activities which are mediated through the same signalling pathways (Demehri, Turkoz and Kopan, 2009; Alspach *et al.*, 2014., Procopio *et al.*, 2015). Senescent CAFs are characterised by developing a senescence-activated-secretory-phenotype (SASP) (Kuilman and Peeper, 2009), with secretion of various growth factors, inflammatory

factors, interleukins, cytokines, ECM proteins and proteases (Coppé *et al.*, 2008, Pazolli *et al.*, 2009). In addition, senescent CAFs play a significant role in promotion of tumour invasion and metastasis as well as resistance of anticancer therapies (Coppé *et al.*, 2008). Furthermore, it has been suggested that senescent CAFs induce EMT in tumours mediated by TGF- β in conjunction with MMP2 that results in down regulation of different cell adhesion molecules (Hassona *et al.*, 2014).

1.3.6 Extracellular vesicles (EVs)

Extracellular vesicles are highly heterogeneous subsets of membrane vesicles secreted by almost all cell types including tumour cells and released in the extracellular spaces. These vesicles vary in their subcellular origin, size and components (figure 1.9). They are considered crucial for regulation of cell to cell communication as well as mediation of extracellular pathways. Many functional molecules are transferred by EVs such as mRNA, miRNA, DNA, proteins, transcriptional factors and lipid, which have the ability to influence the biological functions of recipient cells (Raposo and Stoorvogel, 2013). Furthermore, EVs are categorised according to their biogenesis into 3 groups (Colombo, Raposo and Théry, 2014). Exosomes are the smallest EV type, about 50-150nm in diameter. They form from the release of multivesicular bodies (MVB) containing intraluminal vesicles by exocytosis (Fujita *et al.*, 2015). Microvesicles are larger than exosomes with a diameter ranging from 100-1000nm and are produced by budding or shedding from the plasma membrane (Raposo and Stoorvogel, 2013). Apoptotic bodies are the largest type of EVs about 500-4000nm in diameter (Fujita, Yoshioka and Ochiya, 2016), which contain nuclear fragments (DNA and RNA fragments) as well as cell organelles (Holmgren *et al.*, 1999). They are cleared by macrophages by phagocytosis (Elmore, 2007). It has reported that apoptotic bodies contribute to intercellular communication in the tumour microenvironment (Bergsmedh *et al.*, 2001).

1.3.6.1 Roles of extracellular vesicles (EVs) in the interactions between cancer cells and stromal fibroblasts

EVs have the potential to influence the crosstalk between cancer cells and surrounding fibroblasts in the tumour stroma to enhance tumour progression (Naito *et al.*, 2017). TGF- β enriched cancer derived-EVs mediate the induction of CAFs by activation of fibroblasts (Webber *et al.*, 2010; Webber *et al.*, 2015). Webber and his colleagues (2010) found that cancer derived-EVs were loaded with TGF- β on their surface which stimulated TGF- β /SMAD3 signalling in fibroblasts resulting in induction of a CAF phenotype with expression of α -SMA and FGF2. Moreover, motility and metastasis of cancer cells were enhanced by CAF-derived EVs through activation of the Wnt-Planer cell Polarity signalling pathway (Luga *et al.*, 2012). It has been reported that CAF derived EVs mediate anticancer therapy resistance in cancer cells. For instance, CAF derived EVs that contain non-coding RNA induce Notch target genes leading to enhanced treatment resistance of cancer initiated cells (Boelens *et al.*, 2014). Jossen and his colleagues (2015) revealed that EMT was initiated by secretion of miR-409 contained within CAF derived EVs. In addition, the metabolic properties of both cancer cells and CAFs were modified due the effects of EVs (Chaudhri *et al.*, 2013). For instance, the metabolic properties of cancer cells in prostate cancer were modified by CAF derived EVs (Zhao *et al.*, 2016). On the other hand, fibroblast consumption of glucose in metastatic sites was altered through the influence of cancer derived EVs on pyruvate kinase in breast cancer (Fong *et al.*, 2015).

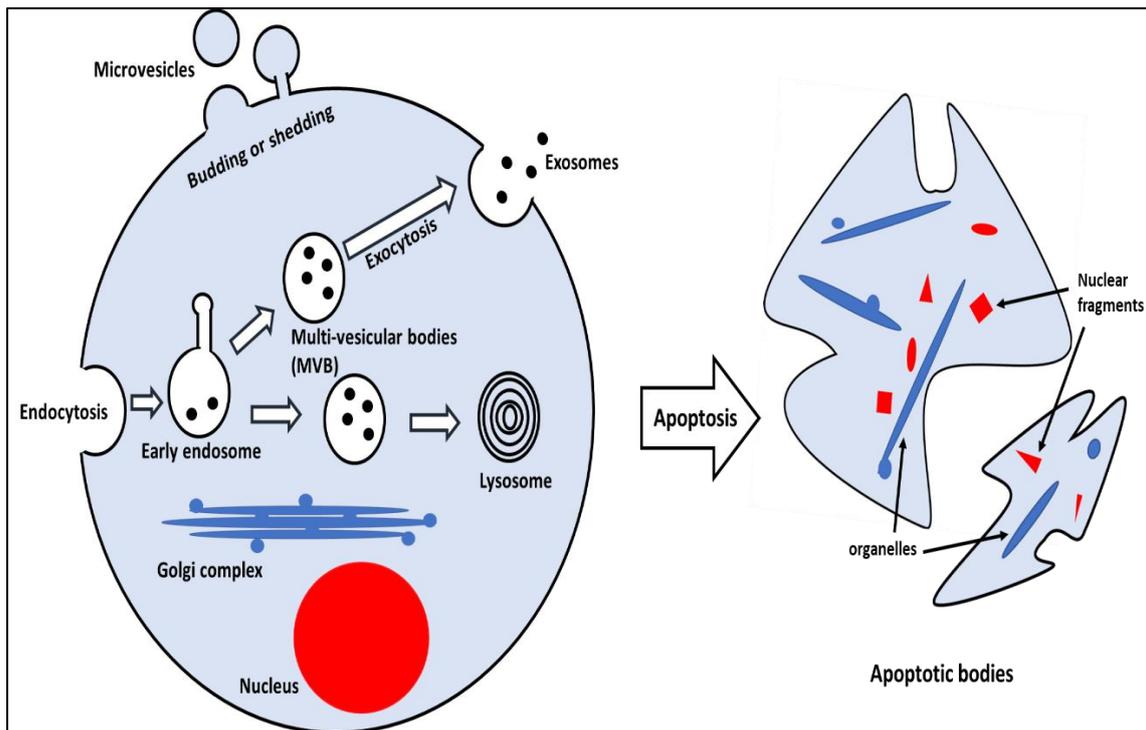


Figure 1.9: Schematic representation illustrating the extracellular vesicles types and biogenesis. Adapted from (Colombo, Raposo and Théry, 2014).

1.3.6.2 Roles of CSC-derived exosomes in the tumour microenvironment

CSC-derived exosomes play pivotal roles in the tumour microenvironment and are considered to be potent regulators of many tumour features. CSC-derived exosomes mediate the complex interactions between monocytes and T-cells. As a result they induce acute tumour immunosuppression through the inhibition of T-cells in the presence of monocytes through their specific secretion of IL-10 (Domenis *et al.*, 2017). CSC-derived exosomes play key roles in mediating and enhancing tumour angiogenesis by different ways. Pro-angiogenic factors such VEGF-A and functional miRNA were secreted by CSC-derived exosomes that resulted in an increased angiogenic potential of endothelial cells as well as promotion of permeability (Treppe *et al.*, 2017). Grange and his colleagues (2011) revealed in their study that renal CSCs with CD105 positive secreted exosomes activated endothelial cells and enhanced the formation of vessels and initiating a pre-metastatic niche for cancer cells. In addition,

angiogenesis was mediated by increasing levels of VEGFR-1 in endothelial cells stimulated by non-coding RNA (lncRNA) H19 release from CSC-derived exosomes (Conigliaro *et al.*, 2015). Sanchez and his colleagues (2015) found that tumour stroma in prostate cancer was modulated by CSC-derived exosomes enriched with miR-139 and miR-183 that resulted in promotion of fibroblast proliferation and migration. Furthermore, it has been identified that CSC-derived exosomes play an important role in tumour stroma remodelling due to their effects on ECM protein synthesis and degradation through mediation of receptor–ligand interactions (Mu, Rana and Zöller, 2013; Sung *et al.*, 2015). On the other hand, CSC-derived exosomes maintain the CSC phenotype by exporting CSC specific molecules such as signalling proteins for the Wnt pathway (Gong and Huang, 2012; de Sousa e Melo and Vermeulen, 2016; Basu, Haase and Ben-Ze'ev, 2016) like β -catenin (Basu, Haase and Ben-Ze'ev, 2016), activators of transcription–Notch pathway component like Jagged, functional enzymes like ALDH and surface receptors such as CD44 and CD133 (Sharghi-Namini *et al.*, 2014; Nakamura *et al.*, 2017).

1.4 Cancer stem cell markers

CSCs markers are cell surface molecules or intracellular soluble proteins which, either alone or in combination have the ability to identify CSCs in various cancers. Therefore, these markers can be used in physical detection and isolation of CSC subpopulations in a tumour from other heterogeneous tumor cells. These markers, whether they are cell-surface or cytoplasmic/nuclear soluble proteins are considered as CSC antigens. However, their expression by the corresponding normal adult stem cells supports the theory that CSCs originate from normal stem cells (Islam, Gopalan, *et al.*, 2015).

It is known that the phenotype of CSCs is not identical in all types of tumours. As a result, there is no unique or universal marker to CSCs (Clarke *et al.*, 2006). However, it has been suggested that a combination of markers may improve the specificity in identification of CSCs (Zhang, Filho and Nör, 2012). For instance, Zhang and his colleagues (2009) isolated CSCs in head and neck cancer using combination of ABCG2, BMI-1, CD44 and Oct4 markers, whereas Han and his colleagues (2014)

used CD44 and CD24. In addition, Zhang and his colleagues (2012) isolated CSCs in oral cancer by using a combination of CD44, CD133 and ALDH. The most common markers used for detection of CSCs of head and neck squamous cell carcinoma are listed in the table 1.1, whereas other important CSCs markers in different cancer types are listed in table 1.2.

1.4.1 Cancer stem cell markers in oral cancer

It has become increasingly clear that using of combination markers for detection of CSCs is tumour type- dependent (Zhang, Filho and Nör, 2012). Therefore, some CSC markers are more relevant to oral cancer than others, which include:

CD44: This is a cell surface glycoprotein involved in cell adhesion and migration, cell-cell interactions, cell signalling and leucocyte attachment and rolling (Ponta, Sherman and Herrlich, 2003; Prince *et al.*, 2007; Takaishi *et al.*, 2009). CD44 is expressed on epithelial cells, erythrocytes and leukocytes (Kemper, Grandela and Medema, 2010) as well as most tumour cells. In normal cells, it has been demonstrated to be a specific receptor for hyaluronic acid that promotes cell migration (Ponta, Sherman and Herrlich, 2003). CD44 acts as a co-receptor of many complimentary receptors on the cell membrane by presenting to them their relevant cytokines and chemokines (Naor *et al.*, 2009). Furthermore, it has affinity for osteopontin and as a result of their interaction, CD44 controls cellular functions that result in tumour progression (Karsten and Goletz, 2013). CD44 is considered one of the most common markers that was used first for identification of CSCs in head and neck squamous cell carcinoma. It has been found that the subpopulation of cells with high expression of CD44 simultaneously overexpressed other specific markers of stemness such as Bmi-1 which maintains the state of undifferentiation of the cell (Prince *et al.*, 2007). CD44 is incorporated into many complex cell signalling cascades which enhance the initiation of tumour through interaction with adjacent receptors such as tyrosine kinases (Wong, Herriot and Rae, 2003). It has been identified that all physiological functions and activities of the CD44 molecule in normal and stem cells were exhibited in cancer cell populations that expressed it (Karsten and Goletz, 2013).

CD24: is a cell adhesion molecule that is bound to the cell membrane through a glycosylphosphatidylinositol (GPI) anchor (Kay, Rosten and Humphries, 1991). In normal physiology, it is expressed on certain epithelial cells, neutrophil and pre-B lymphocytes but it is lost during plasma cell maturation. Therefore, B cell proliferation and maturation was reported as one of the important normal functions of CD24 (Lu *et al.*, 1998). On the other hand, cancer cells in haematological malignancies and several solid tumours expressed CD24 (Kristiansen *et al.*, 2002; Kristiansen *et al.*, 2003) . In head and neck squamous cell carcinoma, it has been reported that CD24⁺/CD44⁺ cells possessed stemness properties and also demonstrate higher invasiveness *in vitro* (Kristiansen, Sammar and Altevogt, 2004; Zhang *et al.*, 2011). Moreover, CD24 is considered to be one of the key ligands of the P-selectin receptor (the main adhesion receptor expressed by activated platelets and endothelial cells) (Nestl *et al.*, 2001). Therefore, it has been established that CD24 strongly related to tumour metastasis due to its role in promotion of adhesion of tumour cells by binding to P-selectin receptors on activated platelets and endothelial cells (Friederichs *et al.*, 2000). Gao and his colleagues (2010) revealed that the CD24 positive cancer cell subpopulations exhibit self-renewal ability and stronger resistance to anticancer therapies compared to CD24 negative cells population. Many studies established CD24 as a marker of CSCs and provide evidence that it plays a crucial role in tumour progression and metastasis when its highly expressed along with CD29, CD31 and CD44 markers in several tumours (Visvader and Lindeman, 2008; Lee *et al.*, 2010).

CD29 (integrin-β1): It is a transmembrane receptor which is part of the integrin receptor family. Integrin receptors consist of an α subunit and β subunit that collaborate in order to bind a specific ECM protein. However, the β1 subunit associates with different α subunits forming various heterodimers each binding to a specific ECM protein depending on α subunit partner and expressing cell type (Hynes, 1992; Brakebusch and Fa, 2003). For instance: α2β1 for collagen, α3β1 for laminin 5 , α5β1 for fibronectin, α8β1 for tenascin and α9β1 for tenascin, fibronectin and vitronectin (Watt, 2002). Integrin-β1 is normally expressed by epidermal keratinocytes with the highest levels in the stem cell of the basal layer than other keratinocytes (Watt, 2002; Braun *et al.* 2003). The normal functions of integrin-β1 involve mediation of adhesion to ECM and initiation of terminal differentiation (Levy

et al., 2000), and motility of cells which is mediated by integrin-mediated adhesion (Schwartz and Assoian, 2001). Moreover, keratinocyte-ECM interactions have a key role in maintaining cell survival, proliferation and the architecture of epidermal tissue (Hynes, 2002). In addition, integrin- β 1 has been implicated in the self-renewal characteristic of stem cells (Lechler and Fuchs, 2006; Taddei *et al.*, 2008) and regulation of their differentiation (Naylor *et al.*, 2001; Watt, 2002). On the other hand, it plays significant roles in tumour progression, migration and invasion. The crosstalk of integrins with oncogenes and growth factor receptors on cancer cells and their associated cells are crucial for tumour growth and invasion. Furthermore, it has been established that integrin- β 1 provides the necessary traction for motility of cells and consequent invasion via its direct binding to ECM proteins as well as its role in regulation of activity of ECM proteases (Assoian and Klein, 2008).

CD133 (prominin-1): Is a glycosylated protein with five transmembrane domains and two large extracellular loops. Normally, it has been reported that CD133 expression is restricted to CD34+ stem cells of the haematopoietic system and plasma membrane protrusion of epithelial cells such as microvilli. Although, till now the functions of CD133 are relatively unknown (Yin *et al.*, 1997). It is thought that CD133 has a role in organization of the topology of plasma membrane (Röper, Corbeil and Huttner, 2000). On the other hand, in head and neck squamous cell carcinoma, CD133 positive stem-like cells have higher clonogenicity, tumourigenesis, invasiveness, and resistance to conventional chemotherapy when compared with CD133 negative cells (Zhang *et al.*, 2010).

Aldehyde dehydrogenase (ALDH) enzymes: ALDH is an intracellular enzyme involved in cell differentiation, detoxification and standard chemotherapy resistance through intracellular aldehyde oxidation (Magni *et al.*, 1996; Sophos and Vasiliou, 2003; Chute *et al.*, 2006). ALDH1 enzyme is considered a marker for the identification of normal somatic cells and CSCs as well as being a prognostic marker to predict tumour prognosis and metastasis (Ginestier *et al.*, 2007; Charafe-Jauffret *et al.*, 2010). In addition, Chen and colleagues (2009) found that radio-resistance and tumourigenicity in HNSCC were enhanced by cell populations with positive ALDH compared to ALDH negative populations.

Oct-4, Nanog and Sox2 transcription factors: These factors play significant roles in the maintenance of self-renewal and pluripotency of embryonic stem cells. In addition, detection of stem cells in the lamina propria of oral mucosa of human adults based on their Oct4, Sox2 and Nanog expression has been reported. Although, the fact that these factors are not expressed on the cell membrane has hindered the effectiveness of CSC sorting by FACS (Boyer *et al.*, 2005; Loh *et al.*, 2006; Chiou *et al.*, 2008). Furthermore, Lim and colleagues (2011) revealed that stem-like cancer cells isolated from HNSCC expressed significantly higher levels of these transcription factors compared to unsorted cells. However, their levels reflect the grade of OSCC, i.e. high grade OSCC has elevated levels of Oct4, Sox2 and Nanog expression (Chiou *et al.*, 2008).

ATP-binding cassette transporters (ABC transporters): These are a superfamily of efflux pumps in the plasma membrane such as MDR1, ABCG2 that determine the side population cell phenotype. They are capable of the efflux of fluorescent DNA dyes like Hoechst 33342 and Dye Cycle Violet (Goodell *et al.*, 1996; Hadnagy *et al.*, 2006). The side population cells in head and neck squamous cell carcinoma that overexpressed ABCG2, Bmi-1, CD44 and Oct4 show cancer stem-like properties with high invasive and metastatic potential (Zhang *et al.*, 2009; Song *et al.*, 2010) .

Bmi-1: is polycomb protein and proto-oncogenic chromatin regulator known to stimulate the self-renewal of stem cells by negatively regulating the expression of Ink4a and Arf tumour suppressors (Chen *et al.*, 2011). In head and neck squamous cell carcinoma Bmi-1 is highly enriched in CD133 positive cells, leading to suppression of apoptosis of these cells and promotion of proliferation (Chen *et al.*, 2011).

Ep-CAM (ESA): Epithelial cell adhesion glycosylated membrane is a protein involved in Wnt signalling and a cancer stem cell surface CD antigen (CD326). It has been reported that two biologically distinct phenotypes of CSC in head and neck squamous cell carcinoma exist based on ESA/CD44 expression: CD44 (high) ESA (high) are proliferative with epithelial characteristics and CD44 (high) ESA (low) are migratory with mesenchymal traits (Laimer *et al.*, 2008).

CD117 (KIT): It is a transmembrane tyrosine-kinase receptor which is part of the platelet-derived growth-factor/colony stimulator factor 1 receptor subfamily. This

receptor is present in various cell types such as haematopoietic progenitor cells, mast cells, germ cells, Cajal cells of the gastrointestinal tract, skin epithelial cells and cerebellar neurons as well as their neoplasms. The KIT receptor is activated normally by binding to stem cell factor which is considered be its specific ligand. Receptor engagement results in activation of signalling cascade leading to activation of different transcription factors involved in cell survival, differentiation, adhesion, chemotaxis and apoptosis regulation (Miettinen and Lasota, 2005).

Table 1.1: Markers relevant to head and neck carcinoma, adapted from (Islam, Gopalan, et al., 2015).

Marker name	Other cancer type with high expression	References
CD44	Breast, colon, stomach, liver, ovary, pancreas, prostate	Ponta, Sherman and Herrlich, (2003), Takaishi <i>et al.</i> , (2009), Prince <i>et al.</i> , (2007)
ALDH Aldehyde dehydrogenase	Breast, colon, liver, pancreas, skin	Sophos and Vasiliou, (2003), Magni <i>et al.</i> , (1996), Chute <i>et al.</i> , (2006)
CD 133 (prominin-1)	Brain, colon, endometrium, liver, lung, ovary, pancreas, prostate, breast	Zhang <i>et al.</i> , (2010)
Oct-4, Nanog and Sox2 Transcription factors	Bladder, breast, liver	Boyer <i>et al.</i> , (2005), Loh <i>et al.</i> , (2006), Chiou <i>et al.</i> , (2008)
ABC transporters	Brain (glioma), gastrointestinal tract, liver, lung, thyroid	Goodell <i>et al.</i> , (1996), Hadnagy <i>et al.</i> , (2006)
Bmi-1	Bladder, skin, prostate, ovary, breast, colon	Chen <i>et al.</i> , (2011)
CD24	Breast, stomach, pancreas	Kristiansen, Sammar and Altevogt, (2004), Zhang <i>et al.</i> , (2011)
Ep-CAM	Colon, pancreas, breast	Laimer <i>et al.</i> , (2008)
CD117 (KIT)	ovary	Sheu <i>et al.</i> , (2005)

Table 1.2: Other important markers of CSCs in various cancer types with their normal functions, adapted from (Islam, Gopalan, *et al.*, 2015).

Marker name	Normal function	Reported cancer	References
CD90	Cell adhesion and signal transduction in T cells	Brain, liver, breast and lung	Yang <i>et al.</i> , (2008), Rege and Hagood, (2006), He <i>et al.</i> , (2012)
CD38	Signal transduction, calcium signalling and cell adhesion	Haematological	Ferrero and Malavasi, (1999)
α 6-Integrin	Cell adhesion, cell-surface mediated signalling	Breast, prostate, brain (glioma)	Hoogland <i>et al.</i> , (2014)
ABCB5	Transmembrane transport of molecules	Colon, skin (melanoma)	Wilson <i>et al.</i> , (2014)
CD26	T cell signal transduction	Colon, leukaemia	Morimoto and Schlossman, (1998), Herrmann <i>et al.</i> , (2014)
CD166	Cell adhesion and cell-cell interactions	Colon, lung	Tachezy <i>et al.</i> , (2014)
LGR5/ GPR49	Cell adhesion	Colon	Walker <i>et al.</i> , (2011)
CD15	Cell adhesion, migration, phagocytosis and chemotaxis	Brain (glioma), Hodgkin's lymphoma	Kerr and Stocks, (1992)
Nestin	Remodelling of the cell	Brain (glioma), prostate	Kleeberger <i>et al.</i> , (2007)
CD13	Regulate peptides and lipid turnover	Liver	Wickström <i>et al.</i> , (2011)
ABCG2	Transport various molecules across extra and intra-cellular membranes	Lung, breast, brain	Bertolini <i>et al.</i> , (2009)
CD20	Regulates B cell differentiation	Skin (melanoma)	Chen <i>et al.</i> , (2005), Fang <i>et al.</i> , (2005)
c-Met	Regulates invasive growth	Pancreas, breast	Furge, Zhang and Vande Woude, (2000)
CXCR4/CD184	Regulates chemotactic activity of lymphocytes	Lung, ovarian	Bertolini <i>et al.</i> , (2009)

Nodal activity	Cell differentiation and signal transduction	Pancreatic	Branford and Yost, (2004)
Keratin 5(K5)	Structural framework of keratinocytes	Bladder, lung	Tokar, Diwan and Waalkes, (2010), Mulvihill <i>et al.</i> , (2013)
P63	Transactivation, regulates apoptosis	Bladder	Di Como <i>et al.</i> , (2002)
CD34	Regulation of cell adhesion	Haematological	Civin <i>et al.</i> , (1984), Lin, Finger and Gutierrez-Ramos, (1995)
E-Cadherin	Cell-cell adhesion	Breast, skin	Alt-Holland <i>et al.</i> , (2008), Dittmer <i>et al.</i> , (2009)
S100A8	Epithelial migration	Colon	Duan <i>et al.</i> , (2013)
Cyclin D1	Induces EMT in CSCs and Epithelial migration	Ovarian, Breast	Wang <i>et al.</i> , (2013)
Ki67	Cell proliferation	Glioblastoma	Li <i>et al.</i> , (2012)

1.4.2 Detection and isolation of CSCs

Identifying and isolating CSCs is considered an important step in order to study their characteristics accurately. There are many methods and techniques used for detection of CSCs, which can be classified into: cell sorting, functional, molecular, image based, filtration and cell adhesion (Islam, Gopalan, *et al.*, 2015) (table1.3).

1. Cell sorting

This approach is characterized by its high specificity and is considered as a reliable method for isolation of CSCs from other non-CSCs based on variety of cell surface and intracellular molecules (markers) (Kentrou *et al.*, 2011). Furthermore, it is subdivided into different techniques:

1.A Fluorescence activated cell sorting (FACS)

FACS is a specialized type of flow cytometry, in which any mixture of heterogeneous biological cells can be sorted on the basis that each cell has a specific scattering of light based on size and granularity (Julius, Masuda and Herzenberg, 1972). When combined with fluorescently labelled antibodies, it can be used to separate and isolate cells with various surface marker phenotypes (Shackleton *et al.*, 2009). The first step of sorting cells from solid tumour includes preparation of a cell suspension through exposing the tumour to enzymes that degrade the cell to cell junctions and attachments to the ECM. The cell suspension is placed in the FACS machine and flowed through a nozzle of a narrow tunnel leading to disruption of the stream and formation of single cell droplets. After that, these droplets are passed through a laser beam which directs certain cells into an electrostatic field due to differences in optical characteristics among cells. The phenomenon of electrostatic deflection bends the flow of charged cells leading to collecting them in the vessel, while the residual electrostatically uncharged cells flow vertically down (Shackleton *et al.*, 2009).

For isolation of certain cells like CSCs, a specific antibody conjugated to fluorescent dye for a certain surface antigen (marker) is incubated with the cell suspension. After that, cells are flowed through the laser beam when is adjusted to the wavelength of the fluorescent dye used and as a consequence to electrostatic deflection, labelled cells can be isolated (Fulawka, Donizy and Halon, 2014).

1.B Magnetic activated cell sorting (MACS).

Antibodies to cell surface CSCs markers used in this method and are bound to magnetic beads. When the cell suspension is incubated with the beads and then exposed to a high magnetic field, the subset of cells that have the specific antigen (marker) will stay in the magnetic column, whereas the rest of the cells can be washed away. After that, the column is removed from the magnetic field and the remaining cells collected (Fulawka, Donizy and Halon, 2014).

2. Functional assays

CSCs have unique properties, so functional approaches have been developed to isolate them from other cells (Podberzin, Wen and Chang, 2012). These methods include:

2.1 Side population assay

This approach is based on the ability of CSCs to efflux dyes and drugs to the outside due to their high expression of ABC membrane transporter proteins. This method includes incubation with Hoechst 33342 dye in suspension followed by FACS sorting (Fulawka, Donizy and Halon, 2014). In FACS analysis, excitation of the Hoechst 33342 dye, results in emission as either Hoechst blue or Hoechst red for non-CSCs tumour cells due to their retention of dye. However, CSCs are negative for both dyes and are considered as side population cells because of their efflux of all Hoechst dyes (Mo *et al.*, 2011).

2.2 Microsphere assay

This assay has been used to evaluate the clonogenic potential of CSCs on the basis of the capability of single CSCs to produce colonies more effectively than their progeny. In addition, these colonies will form spheres when they grow in non-adherent culture. The technique includes plating single cells on soft agar and after 21 days the derived colonies are stained either with crystal violet or nitro-blue-tetrazolium, counted, measured and finally compared to the non-CSCs derived colonies. In general, CSCs demonstrate a larger colony number and size than non-CSCs (Pastrana, Silva-Vargas and Doetsch, 2011).

2.3 ALDH assay

Aldehyde dehydrogenase (ALDH) is an intracellular enzyme that oxidises and converts retinol into retinoic acid leading to detoxification of intracellular aldehydes and is considered important in the resistance of CSCs to chemotherapy (Russo and Hilton, 1988). This assay is based on the characteristic of increased expression of ALDH by CSCs. Therefore, the Aldefluor flow cytometric assay is used to isolate CSCs in various cancer types. It includes addition of the active reagent boron-dipyrromethene (BODIPY) - aminoacetaldehyde to the cells, which is converted later on to BODIPY- aminoacetate by the action of the ALDH enzyme (Hess *et al.*, 2006).

2.4 Xenotransplantation

Xenotransplantation is considered the gold standard in research of CSCs and depends on the tumorigenic features of CSCs and their ability to initiate and develop a secondary tumour similar to original one when they are transplanted to immune-deficient animals (Rosen and Jordan, 2009). In this assay CSCs are isolated using FACS analysis, and then injected either subcutaneously or intraperitoneally into mice. As soon as a secondary tumour develops, the transplantation procedure is repeated. If a tertiary tumour is generated with similar heterogeneous cell population similar to that of primary tumour, it is highly suggestive that the cells isolated first are CSCs (Clarke *et al.*, 2006).

2.5 PKH retention assay

This approach is based on the relative quiescence and slow proliferation features of CSCs and it shows the asymmetric division of these cells. The identification mechanism of retention assay depends mainly on the activity of PKH26 and PKH6 dyes which are lipophilic dyes that irreversibly bind and label the cell membrane. In addition, these dyes are distributed eventually among daughter cells after subsequent cell division. As a consequence, the dye will be diluted through continuous cell division. Hence, non-CSCs have rapid proliferation rate, so they will dilute and lose their membrane dye. On the other hand, CSCs are more dormant with a slow proliferation rate, so they retain PKH dyes for a longer time compared to non-CSC differentiated cells (Pece *et al.*, 2010).

2.6 Chemoresistance assay

CSCs can be isolated on the basis of their resistance to conventional therapy and their survival after treatment. CSCs dose-response curves for chemotherapeutic agents are compared to those for non-CSCs (Tirino *et al.*, 2012).

2.7 Adhesion assay

In this assay CSCs are isolated from other non-CSCs according to their rapid adherence to basement membrane and extracellular matrix molecules such as collagen IV and collagen I compared to the adherence rate of non-CSCs. This rapidity of adhesion of CSCs is attributed to their expression of high levels of specific adhesion molecules like integrins (Jones and Watt, 1993).

3. Molecular assays

Molecular assays have been used to isolate CSCs on the basis of analysis of the expression of specific genes such as stemness genes and transcriptional factors like OCT4, Sox2, and Nanog (Lianidou and Markou, 2011; Tirino *et al.*, 2012). The most sensitive and specific molecular assays are Reverse transcription-polymerase chain reaction (RT-PCR) and quantitative reverse transcription-polymerase chain reaction (RT-qPCR). These assays detect the CSCs through amplification of complementary DNA (cDNA) sequences that identify the target gene depending on the design of oligonucleotide primer probes (Loberg *et al.*, 2004).

4. Image- based approaches

These techniques include: image microscopy, immunocytochemistry and immunohistochemistry and identify CSCs on the basis of site and level of protein markers expression. Moreover, they involve using of fluorescently labelled antibodies against specific antigens in tracked cells, and then the labelled cells are visualized using a fluorescence microscope (Lianidou and Markou, 2011).

5. Filtration

Filtration methods are new techniques which are used for isolation of CSCs, but they need more clinical validation. They include two techniques: micro-filter and micro-chips. In micro-filter cells are isolated depending on their size using specially designed micro-filters (Zheng *et al.*, 2011), while for micro-chips the separation procedure depends mainly on the interaction between the antibody-coated chip surfaces with targeted cells (Stott *et al.*, 2010).

Table 1.3: advantages and disadvantages of different CSCs isolations methods, adapted from (Islam, Gopalan, *et al.*, 2015).

Approach	Method	Advantages	Disadvantages	References
Cytological sorting methods	Fluorescence activated cell sorting (FACS)	Able to isolate and quantify rare cell, multi-parameter separation	Processing may cause artefact and bias cell analysis, limited to cell suspension	Greve <i>et al.</i> , (2006), Cassens <i>et al.</i> , (2002), Panchision <i>et al.</i> , (2007)
	Magnetic-Activated Cell Sorting (MACS)	Easy and fast	Isolate the cells on basis of single parameter, suitable for cell suspension only	Moghbeli <i>et al.</i> , (2014)
Functional assays	Microsphere assay	Simple and easy	Unable to detect quiescent CSCs and has potential to be biased	Pastrana, Silva-Vargas and Doetsch, (2011)
	ALDH assay	Highly stable	Low specificity	Moghbeli <i>et al.</i> , (2014), Yu <i>et al.</i> , (2011)
	SP assay	Easy and simple	Condition dependent, costly, low specificity, lack of purity	Moghbeli <i>et al.</i> , (2014), Mo <i>et al.</i> , (2011)
	PKH retention assay	Easy and widely useful	Low specificity	Tirino <i>et al.</i> , (2012) Pece <i>et al.</i> , (2010)
	Therapy resistance assay	Fast and simple	Low specificity, sensitivity	Tirino <i>et al.</i> , (2012)
	Xenotransplantation	Widely accepted	Potential to be biased	Fulawka, Donizy and Halon, (2014)

Molecular assays	RT-PCR	Flexible, multiplex assay, sample, time and cost effective	Does not allow accurate CSC detection in sample morphological analysis	Reinholz <i>et al.</i> , (2005)
	RT-qPCR	High sensitivity and multiplex analysis	No morphological analysis	Stathopoulou <i>et al.</i> , (2006), Obermayr <i>et al.</i> , (2010)
Image-based methods	Image microscopy	Multi-parameter separation, Highly effective	Time consuming, has potential to be biased	Lianidou and Markou, (2011), Progatzky, Dallman and Lo Celso, (2013)
	Immunocytochemistry	Broad-based and powerful method, relatively inexpensive, very specific	Cross-reactivity, background, qualitative only	Schadendorf <i>et al.</i> , (2003)
	Immunohistochemistry	Inexpensive, highly specific	Limited to tissue sample, need well trained pathologist	Bunea and Zarnescu, (2001)
Filtration	Micro-filter	Time effective and specific	No morphological analysis and needs more clinical validation	Lin <i>et al.</i> , (2010) Zheng <i>et al.</i> , (2011)
	Micro-chips	Time and sample effective	Needs more clinical validation	Nagrath <i>et al.</i> , (2007), Stott <i>et al.</i> , (2010)

1.5 Hypothesis and aims

1.5.1 Background

A study by Locke and his colleagues (2005) showed that there is a small subset of cells within a tumour that drives the growth and other aggressive behaviours of tumour such as tumourigenicity, resistance to therapy, invasion and metastasis. Furthermore, identification and targeting of these cells is considered a crucial step to eradicate the tumour and prevent its recurrence. The primary focus of this study was the morphologic heterogeneity present in different cancerous epithelial cell lines and to determine if this heterogeneity is due to the asymmetric division hierarchies of CSCs or not. (Locke *et al.*, 2005).

The study showed that all examined cell lines demonstrate obvious clonal heterogeneity with a range of colony morphologies of named holoclones, meroclones and paraclones of normal keratinocyte cells (Locke *et al.*, 2005). Moreover, they found that cell proliferation rate was high in the meroclones and early paraclone colonies which may indicate that they contain of early- and late-amplifying cells while the holoclones were composed of smaller, rapid adherent and highly clonogenic cells. Holoclones have the ability to generate all types of colony and are therefore were considered as the source of remaining cells of tumour. In addition, stem cell markers like $\beta 1$ integrin, E-cadherin, B-catenin, CD44 and ESA were expressed at higher levels in holoclone cells than meroclone cells (Locke *et al.*, 2005).

Liang and her colleagues (2014) suggested a method for isolation of CSCs on the basis of their adherence properties rather than clonogenicity or defined marker expression. They describe a non-invasive, low-cost alternative to isolation of CSCs by FACS from oral squamous cell carcinoma. The study involved investigation of primary cells derived from patients with oral squamous cell carcinoma as well as several cell lines from human head and neck squamous cell carcinoma and oral dysplasia. Assays were performed on these cells to allow them adhere to collagen IV and classify isolated cells as rapid adherent for the cells that adhere within 10 minutes, middle adherent cells for that adhered within 30 minutes and finally late adherent for the cells that adhere in 4 hours. After that, further investigations on each group of cells were done to study their characteristics. Firstly, colony and sphere formation assays were used to examine their morphology. Secondly, molecular profile assays were used to investigate stem cell properties. Finally, tumourigenic capacity

was assessed in a tongue xenotransplantation assay in immune-deficient NOD-SCID mice (Liang et al., 2014). The study showed that rapid adherent cells were smaller, more homogenous and had a significant higher colony and sphere forming, with greater migratory ability and higher expression of stem cell markers such as integrin β 1 as well as they displaying a higher tumour initiation ability compared to middle and late adherent cells (Liang *et al.*, 2014).

These two studies suggest that CSC populations exist in OSCC cell lines and that they can be isolated using adhesion assays. This forms the basis of the project described here.

1.5.2 Hypothesis

We hypothesise that cancer stem cells can be isolated from oral cancer cell lines using two different functional assays (cell adhesion and chemoresistance) and that these cells have a stable phenotype, may represent the same population and generate aberrant signalling to surrounding stromal cells.

1.5.3 Aims

- Develop functional assays (adhesion and/or chemoresistance) to isolate cells from OSCC cell lines and investigate the stem cell characteristics of these cells.
- Investigate the stability of the isolated adherent and/or chemoresistant phenotype.
- Investigate the chemoresistance of the early adherent population and the adhesion rate of chemoresistant cells to determine whether cells with similar characteristics are being isolated using the two functional methods.
- Determine whether soluble factors from the CSCs signal differently to stromal cells (fibroblasts) than non-CSCs.
- Investigate whether expression of CSC and fibroblast activation markers correlate in samples of OSCC tissue.

CHAPTER 2

MATERIALS & METHODS

2.1 Cell lines

All cell lines were obtained from the Academic Unit of Oral and Maxillofacial Pathology in the School of Clinical Dentistry, University of Sheffield. All reagents were from Sigma, UK unless otherwise stated.

2.1.1 H357 cell line

Oral keratinocyte H357 cell line originally derived from the tongue of a 74 year old male patient who had a well differentiated (grade II) oral squamous cell carcinoma (provided by Professor S. Prime, University of Bristol) (Prime *et al.*, 1990). This cell line was cultured in keratinocyte growth media (KGM) which consists of: Dulbecco's modified eagles medium (DMEM) and HAMS-F12 medium (3:1), 10% foetal bovine serum (FBS), Penicillin (100 IU/mL) and Streptomycin (100 mg/mL), Amphotericin B (2.5 µg/mL), all from Invitrogen, UK, L-Glutamine (2 mM), Adenine (0.18 mM), Hydrocortisone (5 µg/mL), Cholera toxin (1nM), Insulin (5 µg/mL), and Epidermal growth factor (10 ng/mL).

2.1.2 SCC4 cell line

SCC4 cells are a human squamous cell carcinoma cell line with an epithelial-like morphology. It is derived from the tongue of a 55 year old male patient (Rheinwald and Beckett, 1981). The growth media that used for SCC4 cell line was Dulbecco's modified eagles medium (DMEM) and HAMS-F12 medium (1:1), 10% FBS, Penicillin (100 IU/mL) and Streptomycin (100 mg/mL), Amphotericin B (2.5 µg/mL).

2.1.3 NOF cells

Primary normal oral fibroblasts were derived from gingival biopsies obtained with informed, written consent from patients during elective oral surgery undertaken at the Charles Clifford Dental Hospital, Sheffield, UK, under the ethical approval (number 15/LO/0116) granted by the Sheffield Research Ethics Committee. NOFs were cultured in fibroblast growth media (FGM), which consisted of DMEM (Gibco[®], UK) supplemented with 4.5 g/L D-glucose, 10% (v/v) FBS, Penicillin (100 IU/mL) Streptomycin (100 mg/mL) and Amphotericin B (2.5 µg/mL).

2.2 Cell culture

All cell culture procedures were performed in a class II laminar flow hood. Hood surfaces were decontaminated before and after work sessions using 70% ethanol.

The cell lines were routinely grown usually in 12mL of their specific growth media in 75cm² flasks with filter caps (Greiner Bio-One Ltd, UK) and were maintained in an incubator under controlled conditions at 5% CO₂ and 37°C temperature, until they became approximately 80-90% confluent.

2.2.1 Cell thawing from liquid nitrogen

The cryovial containing the desired cell line was removed from liquid nitrogen container and thawed in a water bath at 37°C with appropriate safety protection. The cryovial contents were then transferred to a centrifuge tube and 2mL of the respective growth media was added. After that, the suspension was centrifuged at 179 g for 5 minutes, the supernatant was removed and the pellet was re-suspended in 1mL fresh respective growth media and transferred to a 75cm² flask. Afterwards, 12mL media was added and the flask incubated in 5% CO₂ at 37°C.

2.2.2 Passaging of cells

Sub-culturing of cells was performed twice weekly to maintain cell viability. Medium was aspirated from the flask using a 10mL serological pipette, then cells were washed twice with phosphate buffered saline (PBS) (Sigma-Aldrich, Dorset, UK) without Mg²⁺ or Ca²⁺. After that, 1mL of 0.05% (v/v) porcine trypsin / 0.02% (v/v) ethylene diamine tetra-acetic acid (EDTA) was added and the flask incubated at 37°C for 10 minutes. Then, by gentle agitation, the cells were detached from the flask wall and this was monitored by microscopic examination. Approximately 3mL of growth medium was then added to the flask to neutralize the enzymatic activity of trypsin, and the suspension was transferred to a tube and was centrifuged at 179 g for 5 minutes. The supernatant was discarded and the cell pellet was re-suspended in fresh growth medium and then transferred to new flasks.

2.3 Adhesion assay

The first step of the adhesion assay (figure 2.1) is coating of a 96-well tissue culture plate (Greiner Bio-One Ltd, UK) with extracellular matrix (ECM) protein. The ECM proteins collagen I and plasma fibronectin (Sigma-Aldrich) were prepared by diluting in sterile PBS to the desired concentration (e.g. 10 µg/mL). Then, 100µL of the ECM solution was pipetted into the required wells of a 96 well plate and the lid labelled accordingly. If there are any uncoated control wells, 100µL PBS alone was added. The plate was then incubated for 1hr at 37°C (or at 4°C overnight). After that, the ECM was removed from the plate and non-specific binding sites were blocked by pipetting 100µL blocking buffer into each well and the plate incubated for 1 hour at 37°C. The blocking buffer was prepared by mixing serum free medium (DMEM: F12, 3:1) and 1% (10 mg/mL) bovine serum albumin (BSA) which was filter sterilised to allow storage in the fridge for a few weeks. During this incubation time, cells were prepared and counted by removing media from the flask, washing the cells with phosphate buffered saline (PBS without Ca²⁺ and Mg²⁺) and trypsinising as described above. Prolonged trypsinisation may damage the protein-related structures of the cell i.e. receptors. Other detachment solutions can be used to detach cells such as Accutase. The cell suspension was transferred into a tube for centrifugation (179 g, 5 mins). Cells were re-suspended in 2-5mL serum free medium (SFM) and counted manually using a light microscope and haemocytometer slide with Trypan blue exclusion (dead cells were stained blue while viable cells did not take-up the dye). The counting procedure involved transferring a mixture of 5µL Trypan blue solution 0.4% (Sigma-Aldrich, UK) and 5µL of cell suspension into the chamber of a haemocytometer slide with a cover slip. The number of viable cells per mL = total number of cells in four squares of haemocytometer slide × dilution factor × 10⁴ / 4.

The next step, cells were seeded into wells by removing the blocking buffer from the plate. 100µL of a 400,000 cells/mL solution was added and the plate incubated for various times up to 1hr at 37°C (this time can be changed). Following the incubation period unattached cells were removed from the plate by inversion. And the wells were washed twice with 100µL SFM. Then, 100µL of SFM was added to each well followed by 20µL of MTS solution (CellTiter 96® Aqueous One Solution Cell Proliferation Reagent in the dark Promega, Southampton, UK). The reagent contains a

tetrazolium compound, 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) which is metabolically cleaved by viable cells. It is light sensitive so the cell culture hood light was turned off when adding the solution. In the final step, the absorbance was measured at 492nm by using of an Infinite® M200 Pro Series spectrophotometer (Tecan UK Ltd) and analysed using Magellan TM Software (Tecan UK Ltd). The results were saved into an Excel sheet document.

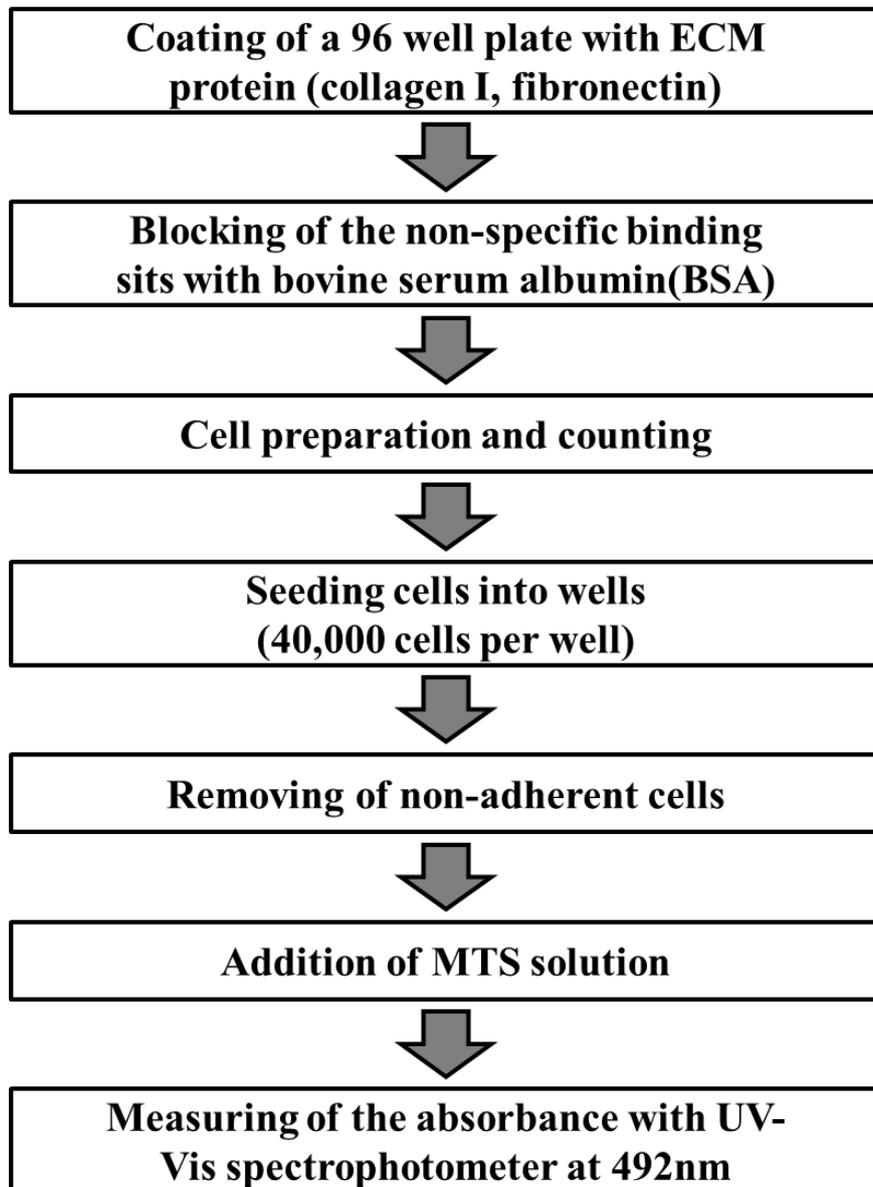


Figure 2.1: Diagram summarizing the steps of the adhesion assay.

2.4 Determination of the stability of the early adherent phenotype

This experiment consisted of 2 parts performed on separate days. In the first part, a T75 cm² flask was coated with 1ml of (75µg/mL) fibronectin and incubated at 37°C for 1 hour. The flask was then washed with PBS and 1mL of the blocking buffer was added to the flask to block any remaining non-specific binding sites. After that, cells were prepared and seeded densities dependent on the cell line used in the flask which was then incubated at 37°C for 10 minutes. The non-adherent cells were washed twice with SFM, while the early adherent cells were collected and seeded into 96 well plate triplicate (that was previously coated with fibronectin and then incubated with the blocking buffer) at density of 40,000 cells per well as well as unsorted cells were seeded with same density in triplicate and incubated at 37°C for 10 minutes. Then the optical density of the first adhesion assay was measured and compared between early adherent and unsorted cells.

The remaining early adherent and unsorted cells were transferred into separated uncoated flasks. Afterwards, both flasks were incubated for 48 hrs. In the second part of the experiment, another adhesion assay was performed using both flasks separately with cells seeded for 10 minutes to determine the stability of the early adherent phenotype.

2.5 Chemoresistance assay

Cells were seeded in triplicate wells for each dose of cisplatin (*cis*-Diammineplatinum (II) dichloride, Sigma-Aldrich, UK) in a 96 well plate at a density of 100,000 cells per well in their growth medium and incubated for 24 hours 37°C. 10µM of fresh cisplatin was prepared for each experiment by dissolving of 3mg of cisplatin powder in 1mL of SFM followed by filter sterilization. The cisplatin stock solution was diluted to a range of final concentrations in SFM (0.5, 2.5, 5, 10, 15, 20, 30 and 50µM). SFM alone acted as a positive control. Wells were washed with SFM before adding cisplatin to each triplicate wells and incubating for 24 hours 37°C. Then, wells were washed in SFM and 100µl of growth medium was added to each well and incubated for 5 days at 37°C. The final steps were similar to that in adhesion assay and involved washing wells twice in SFM as well as 3 empty wells were as negative control followed by the addition of 100µL SFM and 20µl of MTS solution and incubating for 1hour 37°C. Then, the absorbance at 492nm was measured using an Infinite® M200

Pro Series spectrophotometer (Tecan UK Ltd) and the data analysed using Magellan TM Software (Tecan UK Ltd). Thereafter, drug inhibitory response curve was plotted using Graphpad prism software and IC₉₀ was calculated.

2.6 Determination of the stability of the chemoresistant phenotype

A semi confluent flask of cells was treated with 15 μ M cisplatin in SFM and was incubated for 24 hours at 37°C. The flask was washed twice with PBS, growth medium was added and the flask incubated for 5 days at 37°C. After that, the treated cells were seeded as 2 rows of triplicates along with untreated cells at 100,000 cells per well in 100 μ L of growth medium and the plate was incubated for 24 hours 37°C. The wells were washed twice with SFM and 100 μ L of 15 μ M cisplatin solution was added to one row of both previously treated and untreated cells, while 100 μ L of SFM was added to the other row in triplicate wells of just SFM as a control and the plate was incubated for 24 hours 37°C. Then, the wells were washed twice with SFM and 100 μ L of growth medium was added and incubated for 5 days at 37°C. Afterward, growth medium was removed and replaced with 100 μ L SFM and the absorbance was measured following addition of 20 μ L MTS for 1hour 37°C. Cell viability was expressed as percentage of optical density of treated wells related to their untreated controls (figure 2.2).

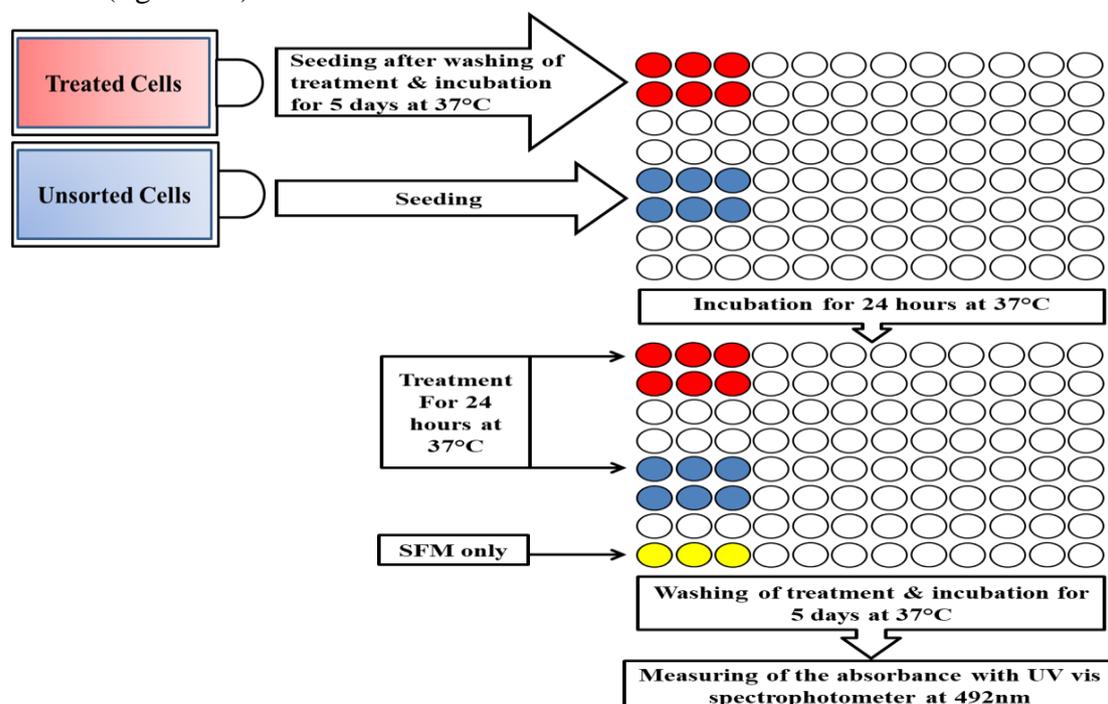


Figure 2.2: Diagram summarizing the steps for the determination of the stability of the chemoresistant phenotype.

2.7 Chemoresistance of early adherent cells

A T75 cm² flask was coated with 1mL of (75µg/mL) fibronectin and incubated for 1 hour at 37°C and then the non-specific binding sites were blocked with 1% BSA for 1 hour at 37°C. After that, unsorted cells were seeded for 10 minutes at 37°C. The non-adherent cells were removed by washing twice with PBS, while the early adherent cells were collected by trypsinisation and seeded into 96 well plate in 2 triplicates at density of 100,000 cells per well as well as unsorted cells at the same density in 2 triplicates. The well plate was incubated for 24 hours at 37°C. Afterwards, the growth medium was removed and one triplicate of both early adherent and unsorted cells were treated with 100µL of 15µM cisplatin in SFM, while SFM only has added to the other triplicate of both conditions as control for 24 hours at 37°C. Wells were washed twice with PBS and 100µL of SFM was added to each well followed by addition of 20µL MTS solution and incubated for 1 hour at 37°C. Finally, the absorbance at 492nm was measured using of an Infinite® M200 Pro Series spectrophotometer (Tecan UK Ltd) and analysed using Magellan™ Software (Tecan UK Ltd) and the percentage of cell viability was calculated and compared between early adherent and unsorted.

2.8 Adhesion of chemoresistant cells

A semi-confluent flask of unsorted cells was treated with 15µM cisplatin in SFM and incubated overnight at 37°C. Then, the flask was washed twice with PBS and growth medium added for 5 days at 37°C. A 96 well plate was coated with 100µL of (75µg/mL) fibronectin and incubated for 1 hour at 37°C and then the non-specific binding sites were blocked with 1% BSA for 1 hour at 37°C. The collected chemoresistant cells and unsorted cells were seeded in triplicate at a density of 40,000 cells per well for 10 minutes at 37°C. Wells were washed twice with PBS, 100µL of SFM is added to each well followed by addition of 20µL MTS solution and incubation for 1 hour at 37°C. The absorbance was then measured at 492nm as described above.

2.9 Proliferation assay

The isolated cells (early adherent cells or chemoresistant cells) and the unsorted cells were seeded in triplicate wells of 4 separate 96 well plates at a density of 10,000 cells in 100 μ L of their growth medium per well and were incubated at 37°C. After every 24 hours (i.e. 24 hrs, 48 hrs, 72 hrs and 96 hrs) one of the plates was removed from incubator. Medium was removed from wells and they were washed twice with DMEM. Afterwards, fresh 100 μ L DMEM was added to each well followed by 20 μ L MTS solution and the plate was incubated for 1 hour at 37°C. The absorbance of each well was measured and growth curves were drawn using the means from each triplicate wells of different days.

2.10 Colony forming assay

Early adherent cells were plated at 1000 cells in 2mL of their growth medium per well in the upper row of a 6 well plate and the same number of unsorted cells per well in the lower row. The 6 well plate was incubated for 14 days at 37°C. Medium were changed every 3 days. After 14 days, the medium was removed and wells were washed with PBS twice. Colonies were fixed in 4% paraformaldehyde in PBS for 30 minutes at room temperature. The fixed colonies were stained with methylene blue stain (5mg/mL) in distilled water for 1 minute. The well plate was washed with distilled water to remove excess stain and the plate was left to dry for 30 minutes at room temperature. Photos were captured and numbers of colonies were counted for each well.

2.11 Flow cytometry for stem cell surface marker expression

Both isolated cells (early adherent or chemoresistant cells) and unsorted cells were detached from flasks using Accutase (BioLegend, UK) for 20 minutes at 37°C. Growth medium was then added and cell suspensions were centrifuged at 179 g for 5 minutes. Cell pellets were re-suspended in flow buffer (PBS containing 10% Foetal bovine serum) and were adjusted to a density of 1x10⁶ cells/1mL. Afterward, samples were prepared by putting 100 μ L (1x10⁵ cells) in each Eppendorf tube on ice. Three direct conjugated antibodies were used in this assay, which were: FITC conjugated

mouse mono-clonal anti- human CD24 (BD Pharmingen™, UK), FITC conjugated mouse mono-clonal anti- human CD29 (integrin-β1) (Life Technologies Ltd., USA) and PE conjugated mouse mono-clonal anti-human CD44 antibody (BD Pharmingen™, UK). Each antibody was added to one sample of isolated cells and one of unsorted cells. In addition, one sample from both types had no antibody added and were used as controls. Amounts of antibodies were added for each sample as the following: CD24 and CD44 were 20µL per 100µL, while CD29 was 5µL per 100µL for 1 hour and the samples on ice in the dark. Ice prevents the antibody complex being internalised into the cells. Then, cells were washed by adding 1mL cold flow buffer to each sample and they were centrifuged at 200 g for 5 minutes. Supernatant was removed and the pellet re-suspended in 200µL of cold flow buffer on ice. In the final step, cell surface expression of CD24, CD44 and CD29 was quantified using a FACS Calibur machine (BD Biosciences, Oxford, UK). In each run 10,000 live cells were assessed and in order to avoid false positive results, gating was performed so that only viable cells were analysed. CellQuestPro Software (BD Biosciences) was used to determine the median fluorescence expression of each stem cell marker. Subsequently, levels were compared between the isolated cells (early adherent or chemoresistant cells) and unsorted cells.

2.12 RNA extraction from isolated and unsorted cells

Extraction of RNA was performed using a RNeasy mini kit (Qiagen) according to manufacturer's instructions. The first step involved lysing the cell pellet in lysis buffer with thorough mixing using a pipette. Afterwards, the lysate was transferred to spin column and 70% ethanol was added and mixed well by pipetting up and down. The lysate was then loaded onto new spin column which contain a silica membrane that binds RNA and this was centrifuged at 11000 g for 30 seconds. Genomic DNA was digested by adding of RNase free DNase 27 K unit/sample to the centre of silica membrane and this was incubated for 15 minutes at room temperature. The membrane was washed for 3 times to remove any impurities such as cellular components and salt metabolites. Finally, the purified RNA was eluted with 50µL RNase-free water by centrifuging at 8000 g for 1 minute and the samples were stored at -80°C.

2.13 cDNA synthesis

Isolated RNA from cells was used to synthesise cDNA using a high capacity cDNA reverse transcription kit (Applied biosystem, Life Technologies Ltd., UK). At first, the concentration of each RNA sample was measured using a NanoDrop 1000 spectrophotometer (ThermoScientific, USA) at 260/280nm and RNA volume was calculated to give 500ng RNA /10 μ L. Reaction master mix was prepared from the kit reagents in accordance with manufacturer's instruction, as shown in the table 2.1. 10 μ L of master mix was added and mixed with each RNA sample. In addition, a negative control sample was prepared by mixing 10 μ L Nuclease free water and 10 μ L master mix. Then, tubes were placed into a thermal cycler (engine DYAD, BIO-RAD, USA) to run a set programme which involved: initially 25°C for 10 minutes, then 37°C for 2 hours and finally at 85°C for 5 minutes. After that, all cDNA samples were stored at -20°C.

Table 2.1: The master mix components used for reverse transcription-polymerase chain reaction (RT-PCR).

Component	Volume/reaction (μ L)	
	Kit without RNase inhibitor	Kit with RNase inhibitor
10 X RT Buffer	2.0	2.0
25 X dNTP Mix (100mM)	0.8	0.8
10 X RT Random Primers	2.0	2.0
MultiScribe Reverse Transcriptase	1.0	1.0
RNase Inhibitor		0.5
Nuclease-free H ₂ O	4.2	3.7
Total per Reaction	10	10

2.14 Real time- Polymerase chain reaction (qPCR)

Two qPCR techniques were used in this study to quantify expression of target genes. First technique was performed using SYBR green probes. The components of the reaction mix are shown in table 2.2.A. The following genes were targeted using primers from Sigma: CD24, CD44 and CD29 for isolated and unsorted cells of oral cancer cell lines and α -smooth muscle actin (α -SMA) for fibroblasts, while the U6 gene was used as an endogenous control. Sequences of these genes for forward and reverse primers are illustrated in the table 2.3. Assays were performed in triplicate on a Rotor-Gene Q real-time PCR machine (QIAGEN, Germany). The thermal cycle consisted of 3 stages: 95°C for 10 seconds, 60°C for 15 seconds and 72°C for 20 seconds for 40 cycles.

The second technique involved using TaqMan probes. The components of reaction mix are shown in the table 2.2.B. This technique was used in this study to investigate the levels of IL-6 (TaqMan, assay ID: Hs00985639_mL, ThermoFisher, USA) gene expression in fibroblasts, whilst the B2M primer (TaqMan, assay ID: Hs00982282, Applied Biosystems, UK) was used as an endogenous control. The same qPCR machine was used as above however, the thermal cycle was different and was composed of 2 stages (95°C for 10 seconds and 60°C for 45 seconds) for 40 cycles.

Rotor-Gene 2.1.0.9 software programme (QIAGEN, Germany) was used to analyse cycle threshold (Ct) values for all samples in both SYBR and TaqMan methods.

The qPCR data was quantified using $\Delta\Delta$ Ct method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008). The relative expression levels of both sorted (early adherent and chemoresistant) and unsorted samples were calculated and compared. At first, the Δ Ct of target gene in each sample was calculated in comparison to its relevant reference housekeeping gene (that reflects the amount of cDNA existed in each sample) to normalize the variation in individual samples, as in the following equations:

$$\Delta\text{Ct sample}_{\text{sorted (early adherent or chemoresistant)}} = \Delta\text{Ct target}_{\text{sorted}} - \Delta\text{Ct reference}_{\text{sorted}}$$

$$\Delta\text{Ct sample}_{\text{unsorted}} = \Delta\text{Ct target}_{\text{unsorted}} - \Delta\text{Ct reference}_{\text{unsorted}}$$

Afterwards, ΔCt values were used for calculation of $\Delta\Delta Ct$ values as in the following equation:

$$\Delta\Delta Ct_{\text{sample sorted}} = \Delta Ct_{\text{sample sorted}} - \Delta Ct_{\text{sample unsorted}}$$

Finally, target gene expression fold change for each sorted sample was calculated as the following:

$$\text{Relative Quantification (RQ)} = 2^{-\Delta\Delta Ct}$$

$$\text{Fold difference} = \log_2(\text{RQ}) = -\Delta\Delta Ct$$

Table 2.2: The components of reaction mixes used in analysis of the gene expression by real time-PCR.

Reagent	Volume (μL)
SYBR Green Master mix	10
Nuclease - free water	7
Forward Primer	1
Reverse Primer	1
cDNA	1
Total	20

(A) SYBR green reaction mix

Reagent	Volume (μL)
TaqMan Master mix	5
Nuclease - free water	3
B2M Primer	0.5
Target Gene Primer	0.5
cDNA	1
Total	10

(B) TaqMan reaction mix

Table 2.3: Sequences of the nucleotide of gene expression primers used for real time-PCR.

CD24	Forward	5' ACAGCCAGTCTCTTCGTGGT 3'
	Reverse	5' CCTGTTTTTCCTTGCCACAT 3'
CD44	Forward	5' CTGCCGCTTTGCAGGTGTA 3'
	Reverse	5' CATTGTGGGCAAGGTGCTATT 3'
CD29 Integrin-β1	Forward	5' AATGAATGCCAAATGGGACACGGG 3'
	Reverse	5' TTCAGTGTTGTGGGATTTGCACGG 3'
α-SMA	Forward	5' GAAGAAGAGGACAGCACTG 3'
	Reverse	5' TCCCATTCACCATCAA 3'
U6	Forward	5' CTCGCTTCGGCAGCACA 3'
	Reverse	5' AACGTTACGAATTTGCGT 3'

2.15 Immunohistochemical staining

Section slides (4µm thickness) of 10 cases of various oral squamous cell carcinoma were obtained from Oral and Maxillofacial Pathology unit, School of Clinical Dentistry under the ethical approval number (07/H1309/150) granted by the Sheffield Research Ethics Committee. The procedure was performed over two days.

On the first day, sections were de-waxed by washing them twice for 5 minutes in xylene (Fisher Scientific, Leicestershire, UK) and they were re-hydrated by putting them in 100% ethanol (Fisher Scientific) twice for 5 minutes each time. Then, slides were incubated for 20 minutes in 3% (v/v) hydrogen peroxide (Fisher Scientific) in 100% methanol (Fisher Scientific). Slides were rinsed with stirring in PBS and antigen retrieval was undertaken by putting slides in 0.01M sodium citrate solution and were subjected to high power in microwave (Panasonic NN-E252W) for 8 minutes. After that they were washed and cooled in PBS. Slides were transferred to a

tray filled with water and blocking of sections was performed using of 100% horse serum at room temperature for 30 minutes afterwards the serum was tipped off. The last step of the first day involved application of diluted primary mouse monoclonal antibodies in horse serum 1/100 v/v (the optimised concentration) and each antibody was added to 3 sections for each case with one section without primary antibody as control. Then slides were incubated a humidified atmosphere overnight at 4°C. The primary antibodies that used in this study:

1. Monoclonal human anti-CD24 antibody 2µg/mL (ab31622, Abcam, UK)
2. Monoclonal human anti-CD44 antibody 0.5µg/mL (ab9524, Abcam, UK)
3. Monoclonal human anti-alpha smooth muscle actin (α -SMA) antibody 10µg/mL (ab7817, Abcam, UK).

On the second day, slides were washed twice in PBS with stirring (using magnet bead at 400 rpm) for 5 minutes each time. Mouse biotinylated secondary antibody VECTASTAIN® Elite ABC-Peroxidase Kits (Vector Laboratories, Peterborough, UK) was prepared by diluting of 1 drop in 10mL of PBS. Secondary antibody was added to slides and incubated for 30 minutes at room temperature. Meanwhile, Avidin Biotinylated enzyme Complex (ABC) reagent (Vector Laboratories) was prepared according to manufacturer's instructions and was left for 30 minutes prior to use. After that, the secondary antibody was tipped off and ABC reagent was added to slides for 30 minutes to enable binding to the secondary antibody. Then slides were washed twice in PBS for 5 minutes each time.

DAB 3,3'-diaminobenzidine tetrahydrochloride (Vector Laboratories) substrate was prepared according to manufacturer's instruction and was added to slides until a dark brown precipitate appeared. The slides were transferred to distilled water to stop the reaction. After that slides were counterstained with haematoxylin using a Leica ST4020 Small Linear Stainer (Leica Microsystems, Milton Keynes, UK) and the steps of this procedure are shown in the table 2.4. The final stage involved mounting slides with cover slips using DPX non-aqueous mounting medium (Thermo Fisher Scientific, UK).

Slides were digitally scanned using Aperio ScanScop (Leica Biosystem, USA) and were archived and viewed in the dentalmicroscopy.shef.ac.uk website using e.slide manager Digital Pathology software (Leica Biosystem, USA). Six images of same magnification were captured for selected equal sized regions of interest (ROI) of the

tumour invasive front regions in each tumour section slide using the Aperio ImageScope software (Leica Biosystem, USA) and a free hand tool of ImageJ software was used to select and analyse the specific ROI in the images to determine the percentage contribution of high, moderate and low density of antibody staining in each image and an average of them was calculated. Antibody staining density for each slide was determined by calculating the average value of the three positive densities for the six images for a specific slide.

Table 2.4: The protocol for counterstaining using a small liner stainer. The duration of each step was 1 minute.

Step	Process
1	Harris's haematoxylin (x2)
2	Running tap water
3	1% (v/v) acid alcohol (1% HCl in 70% isopropanol)
4	Running tap water
5	Scott's tap water substitute (3.5g/L sodium bicarbonate & 20g/L magnesium sulphate)
6	Running tap water
7	95% ethanol (x2)
8	100% ethanol (x2)
9	Xylene (x3)

2.16 Immunofluorescence

Cells were seeded at a density of 5,000 cells on a glass coverslip (Fisher Scientific, USA) in 1mL of their growth medium in a 24 well plate for 48 hours at 37°C. Medium was removed from wells of a 24 well plate that containing cells seeded on coverslips and were washed gently with PBS to avoid removing of cells. Coverslips were fixed with 100% methanol (Fisher Scientific, UK) for 15 minutes, permeabilised with 4mM sodium deoxycholate in PBS for 15 minutes followed by incubation with blocking buffer (2.5mg Bovine Serum Albumin in 100mL PBS) for 30 minutes at room temperature. Coverslips were incubated with 0.2mL of FITC conjugated anti-

alpha smooth muscle actin (α -SMA) antibody (1:100 dilution, Clone A.4, Sigma, UK) for 1 hour at room temperature. They were washed 3 times with PBS. Before mounting using ProlongTM Diamond Antifade Mountant with DAPI (ThermoFisher, USA). Slides were viewed using a fluorescent microscope (Zeiss Axioplan 2 and the image-ProPlus 7.0.1 imaging software Zeiss, Ltd).

2.17 Activation of NOFs by early adherent and chemo-resistant cells

Cells isolated either by adhesion (described in 2.3) or chemo-resistance (described in 2.5) and their relevant unsorted cells were seeded with 1mL of their growth medium in a 24 well plate in triplicate at a density of 200,000 cells per well and incubated at 37°C for 24 hours (figure 2.3). The following day, medium was removed and cells were washed in DMEM twice and 1mL low serum containing medium (0.5% FCS and DMEM: F12 3:1 with antimicrobials) was added and incubated at 37°C for 48 hours. Meanwhile, glass coverslips were placed in a 12 well plate and were sterilised with 70% ethanol. Normal oral fibroblasts (NOFs) were plated on the coverslips containing wells with 1mL of their growth medium and their density was dependent on which assay was used (i.e. 5000 cells per well for immunofluorescence and 100,000 cells per well for qPCR). Following incubation at 37°C for 24 hours, conditioned medium was transferred to labelled Eppendorf tubes which were centrifuged at 300 g for 10 minutes. Meanwhile, medium was removed from the NOFs seeded on coverslips and 1mL conditioned medium from isolated and unsorted cells was added to triplicate NOFs seeded coverslips wells. In addition, low serum medium containing 10ng/mL TGF- β 1 (Human recombinant TGF- β 1, R&D System) was added to triplicate NOFs seeded coverslips wells as a positive control as well as triplicate wells with just low serum medium as a negative control. Afterward the plates containing NOFs were incubated at 37°C for 48 hours.

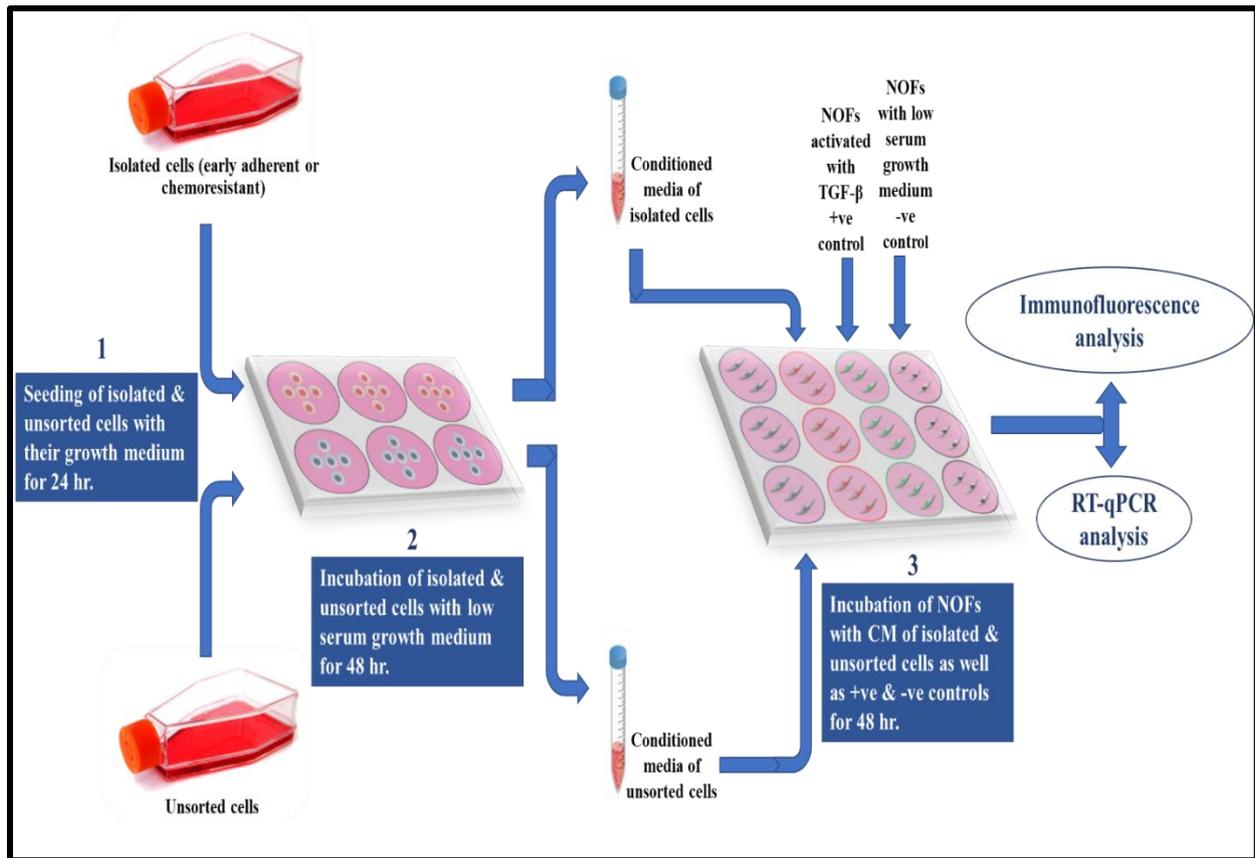


Figure 2.3: Diagram summarizing the steps of the method of activation of NOFs by early adherent and chemo-resistant cells.

2.18 Secretion of extracellular vesicles by early adherent and chemo-resistant cells

Isolated cells (early adherent or chemoresistant cells) and unsorted cells were seeded in 6 well plates with 2mL of their growth medium at 500,000 cells per well and incubated at 37°C for 24 hours. The next day, medium was removed and 1mL low serum containing medium was added and incubated at 37°C for 24 hours. Conditioned medium were transferred from isolated and unsorted cells to labelled Eppendorf tubes and placed on ice. The wells were washed with PBS and 500µL trypsin were added to each well and the 6 well plate was incubated at 37°C for 10 minutes followed by adding of 500µL of low serum medium to each well. The cell suspension was transferred into Eppendorf tubes for centrifugation (300 g, 5 mins). Cells were re-

suspended in 1mL low serum medium and counted using a haemocytometer slide. Serial centrifugations were then performed on the collected condition medium samples. Firstly, they were centrifuged at 300 g for 10 minutes and supernatant of each sample were transferred to fresh labelled Eppendorf tubes. After that they were centrifuged at 2000 g for 15 min, followed by the last spin at 10000 g for 30 minutes. Samples were filtered using a 0.22µm centrifugal filter immediately before Tunable resistive pulse sensing (TRPS). An IZON Qnano machine (IZON Science Ltd., New Zealand) was used to perform TRPS and data analysed using iZON Control Suite software (IZON Science Ltd., New Zealand) to identify the concentration, size and distribution of vesicles in each sample. Finally, the exact number of vesicles per cell that produced by each sample were calculated according to the following equation:

$$\text{Number of extracellular vesicles of each sample} = \frac{\text{vesicles raw count per mL}}{\text{number of cells for each sample per mL}}$$

2.19 Statistical analysis

GraphPad Prism, version 7 software was used for analysis of all comparisons. Testing of statistical was performed using an unpaired two tailed Student's t-test with P value < 0.05. Whereas, liner regression of scatter and Pearson's coefficient were used to test the statistical significance of data correlation.

CHAPTER 3
ISOLATION & STABILITY OF
CSCs PHENOTYPE

3.1 Introduction

As described earlier, oral cancer is a heterogeneous disease (Mannelli and Gallo, 2011), with poor prognosis due to failure of conventional anticancer therapies and a high recurrence rate as well as developing of metastasis and generating of secondary tumours at distant sites (Daniela *et al.*, 2012). All these mentioned causes could be attributed to a small population of cells within a tumour which are called cancer stem cells (Prince and Ailles, 2008). Therefore, developing accurate and effective methods for isolation of CSCs is a crucial step for better understanding of the biological behaviour of these cells in cancer research (Beck and Blanpain, 2013) and subsequently, developing of novel strategies of targeting them (Zhou *et al.*, 2009).

Currently, various techniques are used for isolation of CSCs which are based on: cell sorting, image, molecular, functional and filtration approaches (Milne *et al.*, 2009; Lianidou and Markou, 2011; Tirino *et al.*, 2012; Podberezin, Wen and Chang, 2012). However, fluorescent activated cell sorting analysis (FACS) is considered the most common method that is used to identify and isolate CSCs on the basis of their expression of specific surface markers (Li *et al.*, 2007; Vermeulen *et al.*, 2012). However, FACS has several limitations that influence the accuracy of results. For instance, some extrinsic factors such as calibration of machine, compensation/gating protocol and use of appropriate controls as well as intrinsic factors such as cell confluence and clonal variations. In addition, expression of surface antigens does not necessarily reflect the behaviour of CSCs (Chen *et al.*, 2014). Moreover, it has been reported that interactions of the tumour stroma with cancer cells have a great influence on the stemness properties of CSCs (Chaffer *et al.*, 2011). On the other hand, some recent studies reported that functional methods of CSCs isolation based on their unique intrinsic features such as rapid adhesiveness to ECM proteins represented effective alternatives to FACS (Ling *et al.*, 2014).

Therefore, in this study we aimed to develop non-invasive, highly functional methods to form alternatives to FACS in order to isolate oral CSCs from oral cancer cell lines on the basis of the rapid adherence ability of CSCs to ECM proteins as well as their capability to resist conventional chemotherapies. Furthermore, we investigated the stability of the isolated cell phenotypes in culture several days after their isolation. In

addition, we explored if there is a phenotypic correlation between the populations of cells isolated by the two methods.

3.2 The aims of the chapter

1. To assess the factors that affect the adhesion of cells to ECM proteins in order to develop an effective adhesion assay by which we could isolate the most purified subpopulation of cells with CSC-like properties.
2. To investigate the stability of the adherent phenotype in culture 2 days after the isolation.
3. To assess the required IC_{90} (the conc. of cisplatin that kill approximately 90% of the total number of seeded cells) and isolate approximately 10% viable cells (chemoresistant cells) which are suspected to have CSC-like properties.
4. To investigate the stability of the isolated chemoresistant phenotype after exposing to cisplatin for a second time compared to unsorted cells.
5. To investigate if there is a phenotypic correlation between the isolated subpopulations of cells using the two methods (adhesion and chemoresistance).

3.3 Methods

The following methods were used in this chapter:

- Cell culture (section 2.2)
- Adhesion assay (section 2.3)
- Cell counting (section 2.3)
- Determination the stability of the early adherent phenotype (section 2.4)
- Chemoresistance assay (section 2.5)
- Determination the stability of the chemoresistant phenotype (section 2.6)
- Chemoresistance of early adherent cells (section 2.7)
- Adhesion of chemoresistant cells (section 2.8)

3.4 Results

3.4.1 Assessment of the effects of time, ECM proteins and oral cancer cell line on the adhesion rate and isolation of early adherent cells.

The adhesion of both H357 and SCC4 oral cancer cell lines (OCCL) to collagen I and fibronectin was investigated at different incubation time points from 10 minutes to 60 minutes at 10 minutes intervals.

The results show that adhesion of both oral cancer cell lines is less to fibronectin than that to collagen I at all time points and it is considerably less at 10 minutes. The adhesion to both ECM proteins increases with incubation time (figure 3.1 and 3.3). Maximum adhesion of H357 cells to collagen I was achieved after 30 minutes.

H357 cells adhere to both collagen I and fibronectin at a significantly higher level compared to the control (uncoated wells) at all-time points. Moreover, significantly more H357 cells attached to collagen I than to fibronectin at all-time points figure (3.1 and figure 3.2).

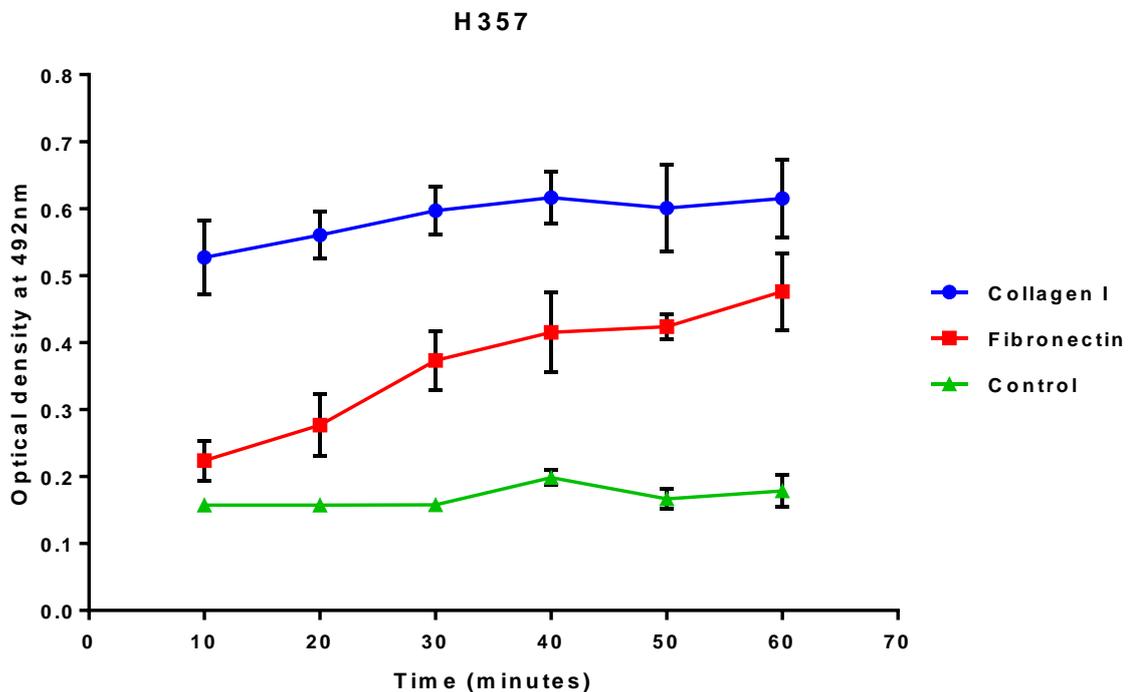


Figure 3.1: Adhesion of H357 cell line to collagen I (blue line), fibronectin (red line) and control (green line) at different time points. All the assays were performed in triplicate and repeated three times. The error bar represents SEM. (p-value <0.0001) indicates the statistical difference of adhesion of H357 cell line to collagen I compared to fibronectin as well as the adhesion to both collagen I and fibronectin compared to control at all time points.

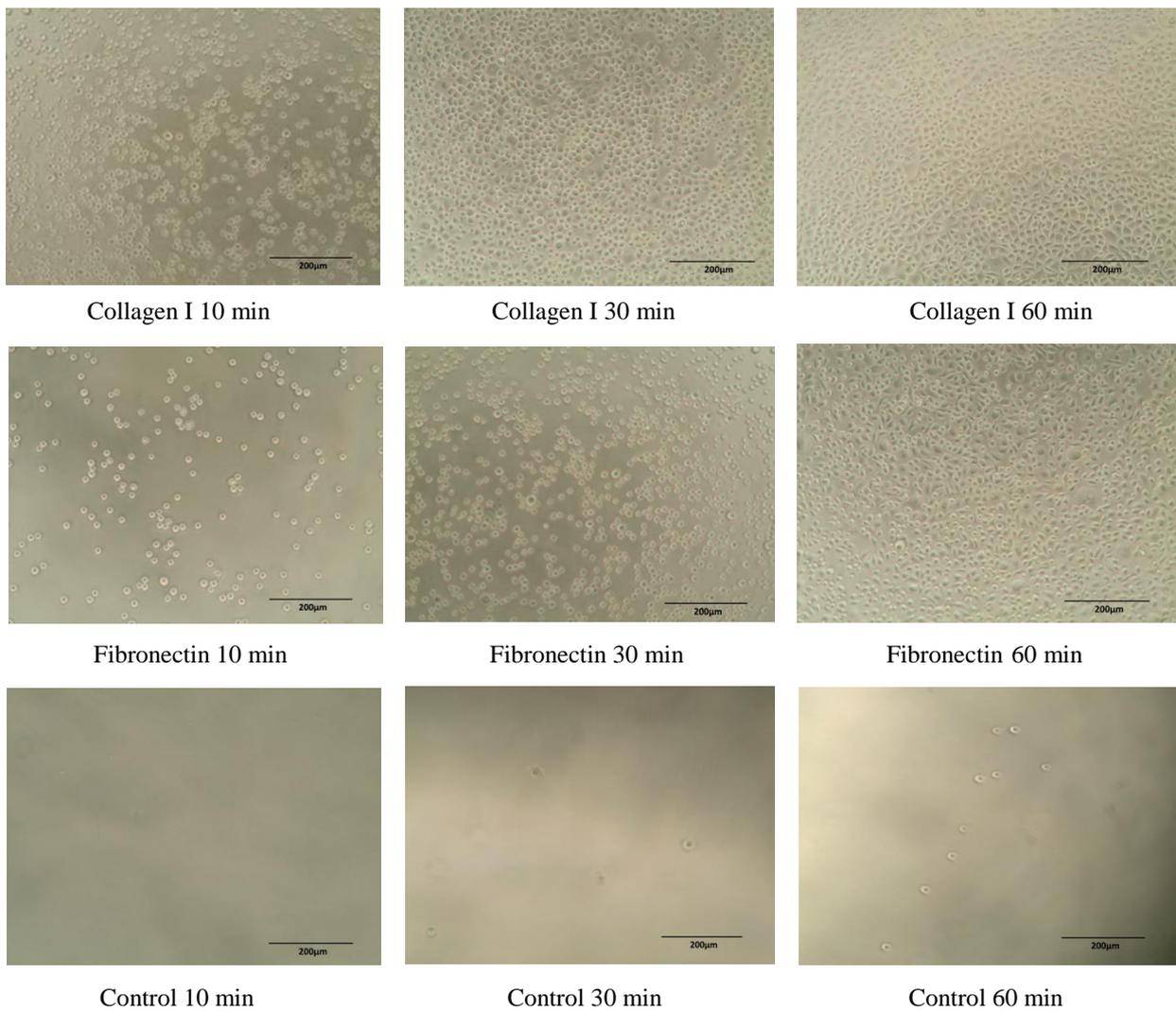


Figure 3.2: Photomicrographs showing the difference in adhesion of the H357 cell line to collagen I, fibronectin and control at different time points. The adhesion of H357 cells to fibronectin is noticeably less than that to collagen I at 10 minutes and the adhesion to both ECM is increased with incubation time. Maximum cell adhesion occurs to collagen I after 30 minutes. No noticeable cell adhesion to control (uncoated wells) was seen at all time points.

The adhesion of the SCC4 oral cancer cell line to both collagen I and fibronectin was significantly higher than control. However, more SCC4 cells adhere to collagen I compared to fibronectin at all-time points (figure 3.3). In addition, the findings demonstrated that SCC4 cell line adheres significantly higher to collagen I and fibronectin than H357 cell line at all-time points.

Our findings showed that the average number of early adherent cells of both oral cancer cell lines to fibronectin at 10 minutes represented less than 10% of the average number of the seeded cells (H357= 4.49% and SCC4= 5.51%), figures 3.4 and 3.5. Therefore, we selected the adhesion of OSCC cell lines to fibronectin at 10 minutes to isolate CSCs in order to obtain a more purified population of CSC-like cells.

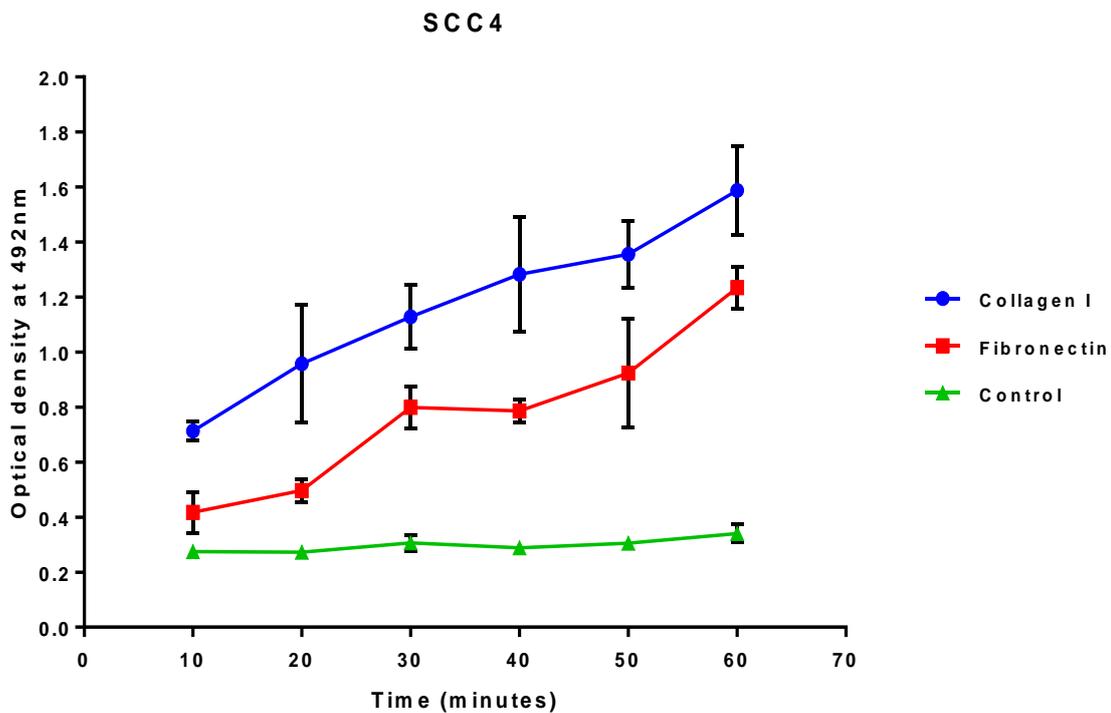


Figure 3.3: Adhesion of SCC4 cell line to collagen I (blue line), fibronectin (red line) and control (green line) at different time points. All the assays were performed in triplicate and repeated three times. The error bar represents SEM. (p-value <0.0001) indicates a statistical difference of adhesion of SCC4 cell line to collagen I compared to fibronectin as well as the adhesion to both collagen I and fibronectin compared to control at all-time points.

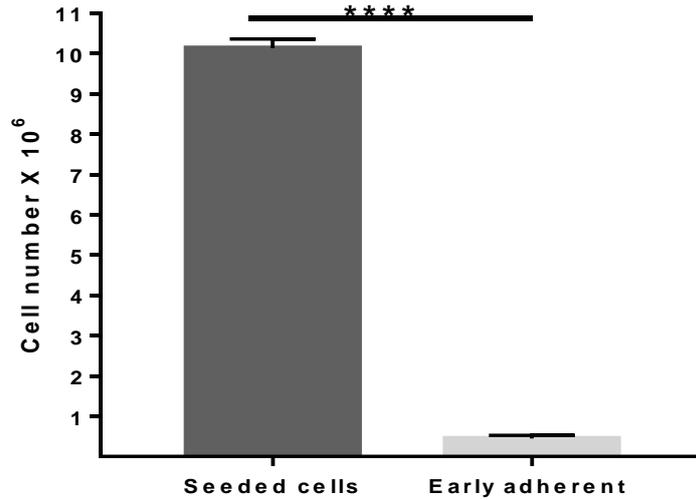


Figure 3.4: The number of early adherent H357 cells to fibronectin at 10minutes time point compared to the total number of seeded cells. The early adherent cell number (light grey column) = 4.49% of the total number of seeded unsorted H357 cells (dark grey column). The error bar represents SEM. P value < 0.0001.

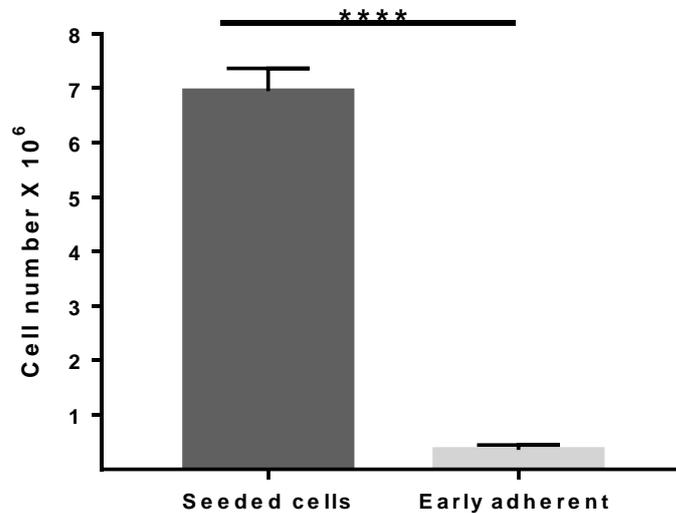


Figure 3.5: The number of early adherent SCC4 cells to fibronectin at 10minutes time point compared to the total number of seeded cells. The early adherent cell number (light grey column) = 5.51% of the total number of seeded unsorted SCC4 cells (dark grey column). The error bar represents SEM. P value < 0.0001.

3.4.2 Effect of cell detachment solution on the adhesion rate

This experiment was performed to investigate if there is an influence of the different types of cell detachment solution on subsequent the cell adhesion. The result showed no significant difference in cell adhesion of the H357 cell line to both collagen I and fibronectin proteins, whether trypsin/EDTA or Accutase was used as a cell detachment solution (figure 3.6).

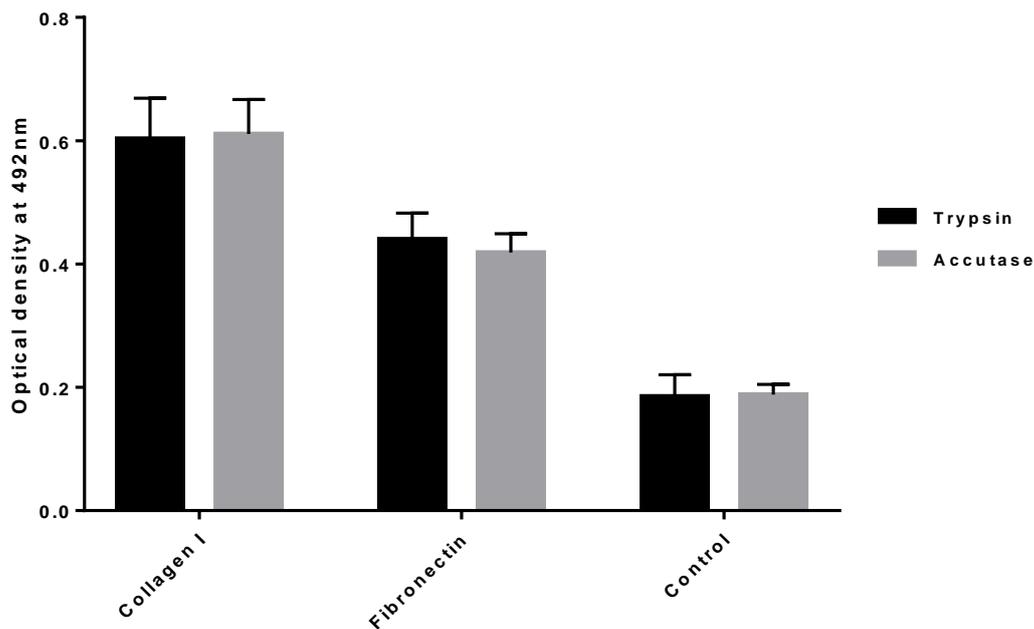


Figure 3.6: The effect of two different cell detachment solutions trypsin and Accutase on the adhesion of H357 cell line to ECM proteins (collagen I and fibronectin). The black columns represent the adhesion of cells detached by using trypsin. The grey columns represent the adhesion of the H357 cell detached by using Accutase. For collagen I (P value = 0.88), Fibronectin (P value = 0.51).

3.4.3 Stability of early adherent phenotype

In these experiments the stability of early adherent phenotype of the two oral cancer cell lines (H357 and SCC4) was assessed by measuring their early adhesion rate to fibronectin after incubation at 37°C for 48 hours.

The early adherent phenotype of both cell lines H357 and SCC4, was conserved to fibronectin after incubation for 48 hours and was significantly higher compared to unsorted cells, (figures 3.7 and 3.8).

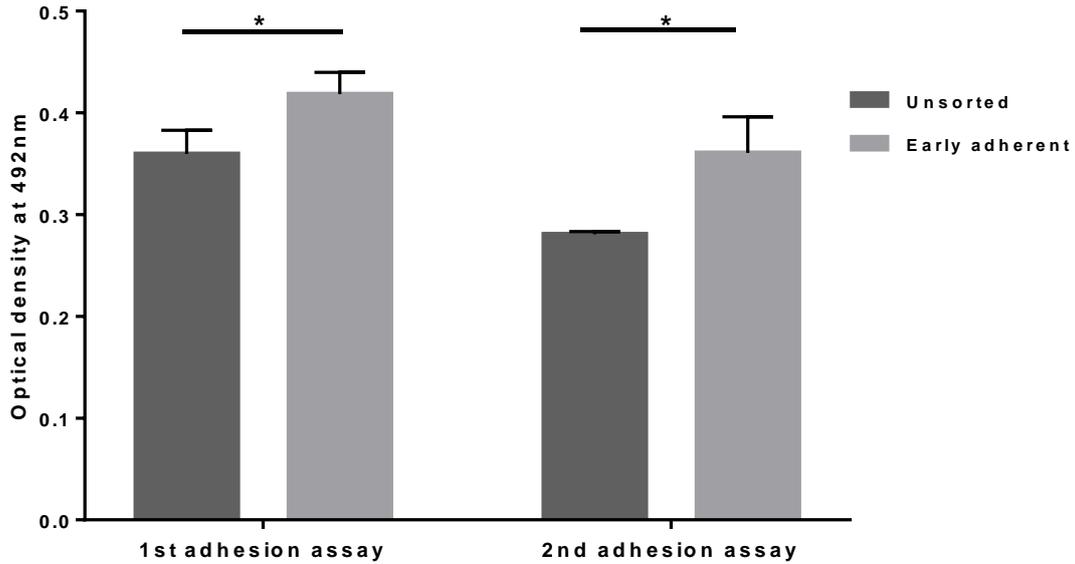


Figure 3.7: Stability of early adherent phenotype of H357 cell line to fibronectin. The dark grey columns represent the adhesion of unsorted cells phenotype, while the light grey columns represent the adhesion of early adherent phenotype. The first adhesion assay was performed by measuring the adhesion after 10 minutes on the same day of cell isolation while the second adhesion assay represented a repeating of adhesion for 10 minutes after 48 hours incubation of both early adherent and unsorted cells in culture. The error bar represents SEM. P value = 0.03 in the first adhesion assay and P = 0.01 in the second adhesion.

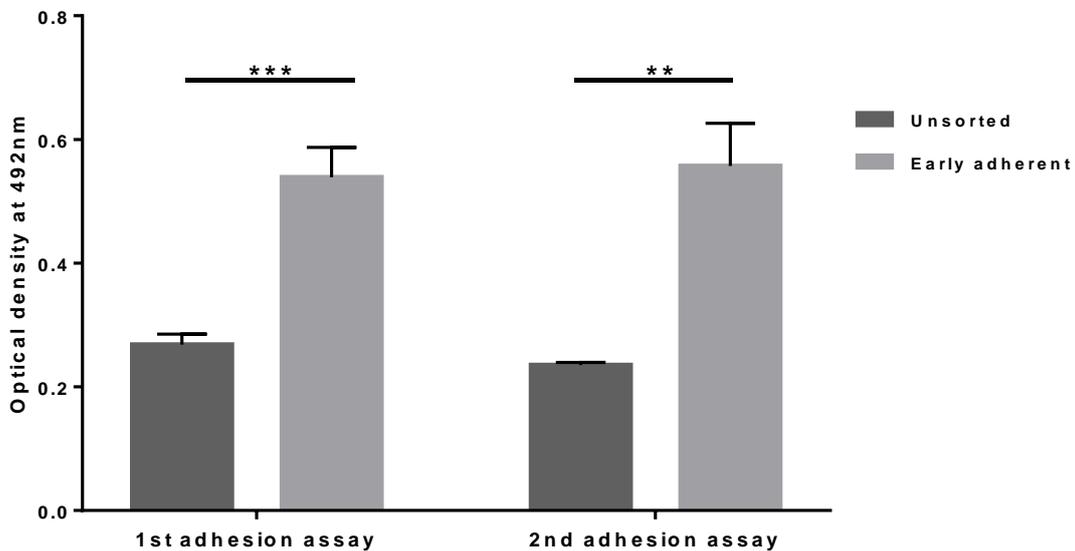


Figure 3.8: Stability of early adherent phenotype of SCC4 cell line to fibronectin. The dark grey columns represent the adhesion of unsorted cells phenotype, while the light grey columns represent the adhesion of early adherent phenotype. The first adhesion assay was performed by measuring the adhesion after 10 minutes on the same day of cell isolation while the second adhesion assay represented a repeating of adhesion for 10 minutes after 48 hours incubation of both early adherent and unsorted cells in culture. The error bar represents SEM. P value = 0.0008 in the first adhesion assay and P = 0.001 in the second adhesion.

3.4.4 Assessment of sensitivity of oral cancer cell lines to cisplatin and isolation of chemoresistant cells.

Cytotoxicity assays were performed using a range of cisplatin concentrations on both OSCC cell lines (H357 and SCC4) and drug inhibitory response curves were plotted to determine the values of IC₉₀ (drug inhibitory concentration 90) which is the concentration of drug that kills 90% of cells, the remaining 10% of cells which were viable were then subjected to further characterisation.

The findings demonstrated that the IC₉₀ of H357 cell line was 15 μ M (figure 3.9), whereas for SCC4 cell line was 17.5 μ M (figure 3.10). Overall there was no significance difference in the sensitivity between the 2 cell lines.

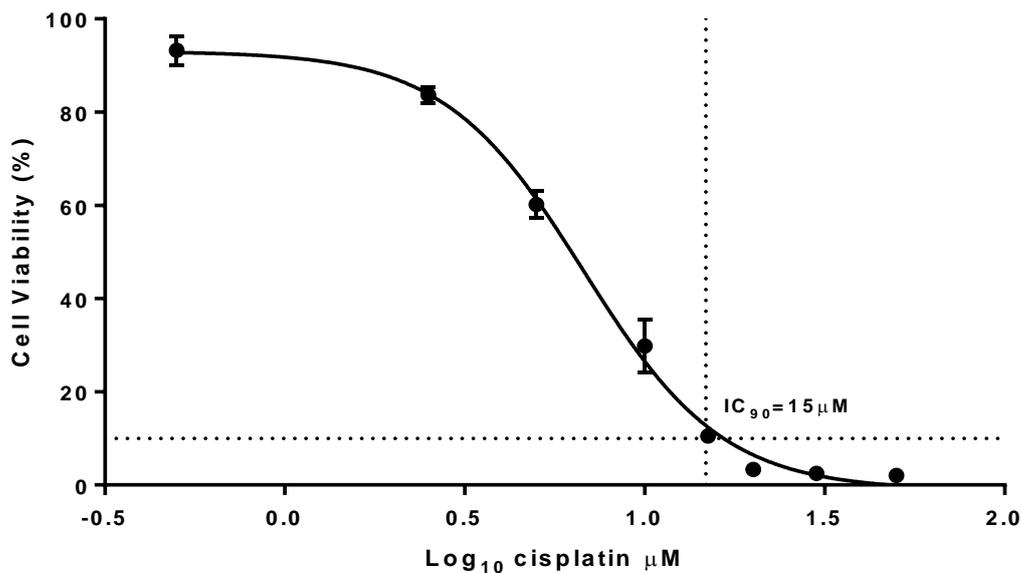


Figure 3.9: Drug inhibitory response curve of H357 cell line to cisplatin. Concentrations of cisplatin were represented by Logarithms and the drug inhibitory curve and IC₉₀ were generated using GraphPad prism software. IC₉₀= 15 μ M.

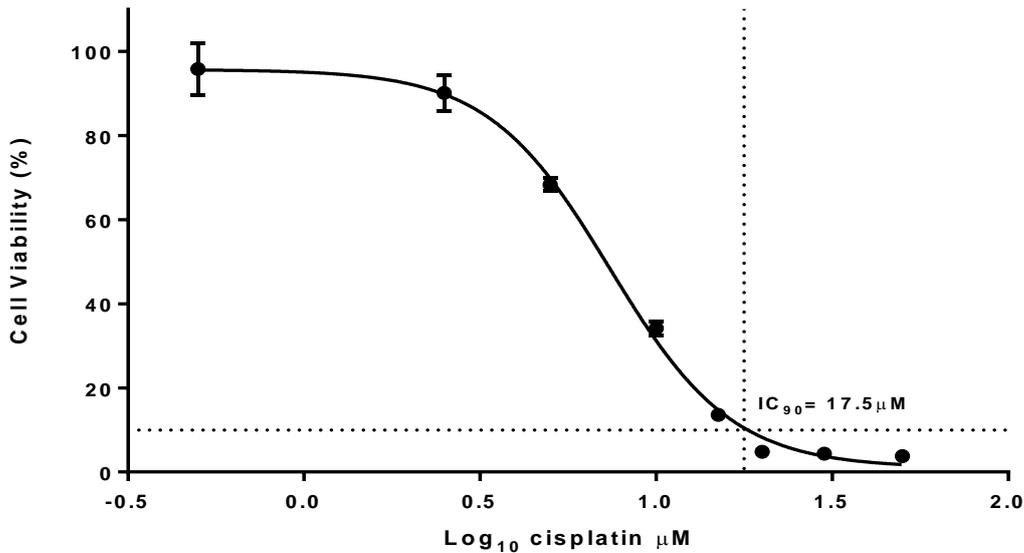


Figure 3.10: Drug inhibitory response curve of SCC4 cell line to cisplatin. Concentrations of cisplatin were represented by Logarithms and the drug inhibitory curve and IC₉₀ were generated using GraphPad prism software. IC₉₀= 17.5μM.

The average number of viable H357 cells after treatment using the previously determined IC₉₀ concentrations represented 8.7% of the average total number of seeded cells before treatment, while for SCC4 it was 10.2%, figure 3.11.

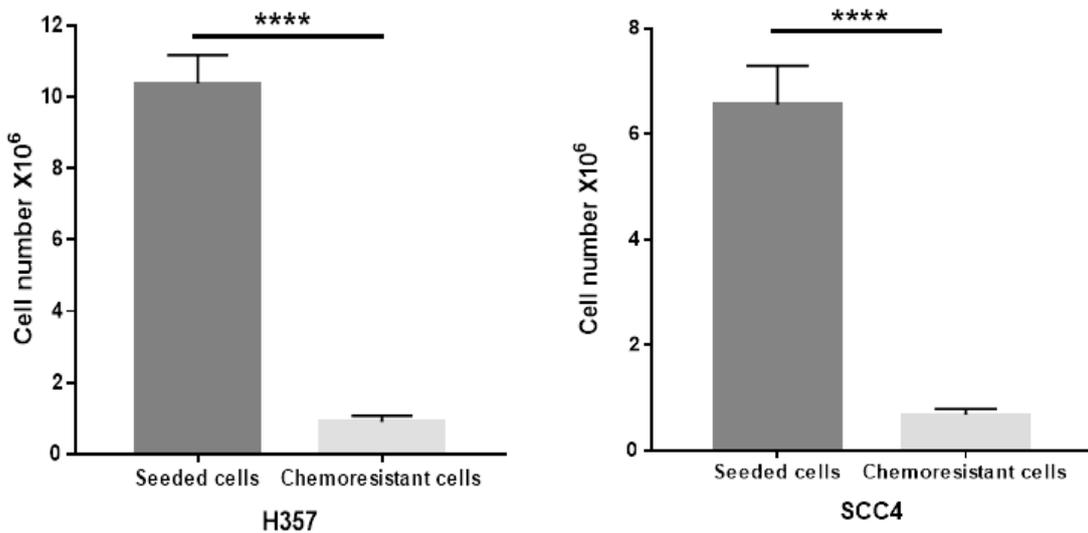


Figure 3.11: The number of chemoresistant H357 and SCC4 cells compared to the total number of seeded cells before treatment. The light grey columns represent the number of chemoresistant cells which presented for H357= 8.7% and for SCC4=10.2% of the total number of seeded unsorted cells which are represented by the dark grey columns. The error bar represents SEM. P value < 0.0001.

3.4.5 Stability of chemoresistant phenotype

The stability of the chemoresistant phenotype was determined by exposing previously treated chemoresistant cells to the same concentration of cisplatin again and comparing their viability to that of untreated cells that had not been previously exposed to the drug.

The results showed that was significantly higher levels of viability of chemoresistant cells of both OSCC cell lines (H357 and SCC4) in comparison to untreated cells, figures 3.12 and 3.13.

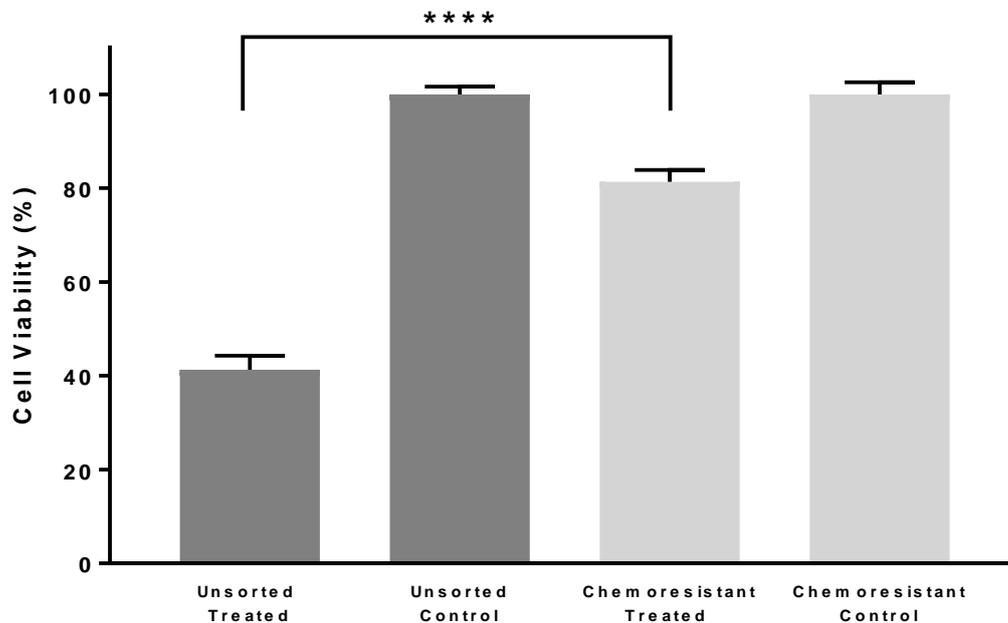


Figure 3.12: Stability of the chemo-resistant phenotype of H357 cell line after re-treatment compared to untreated unsorted cells. The dark grey columns represent the cell viability of untreated unsorted cells phenotype, while the light grey columns represent the cell viability of chemoresistant phenotype. The error bar represents SEM. P value < 0.0001.

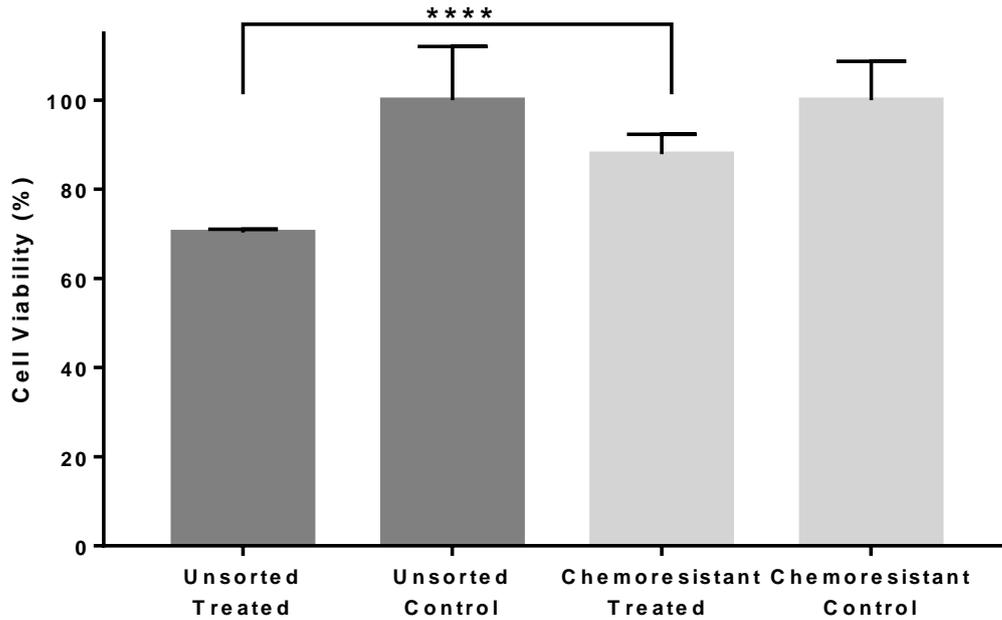


Figure 3.13: Stability of the chemo-resistant phenotype of SCC4 cell line after re-treatment compared to untreated unsorted cells. The dark grey columns represent the cell viability of untreated unsorted cells phenotype, while the light grey columns represent the cell viability of chemo-resistant phenotype. The error bar represents SEM. P value < 0.0001.

3.4.6 Determination the chemoresistance of early adherent cells

In order to investigate the chemoresistance capability of early adherent cells, they were treated with 15 μ M cisplatin. Then cell viability was calculated and compared of both adherent cells and unsorted cells.

The early adherent H357 and SCC4 cells exhibited higher levels of resistance to the chemotherapy with significantly increased levels of viability than unsorted cells. (figures 3.14 and 3.15).

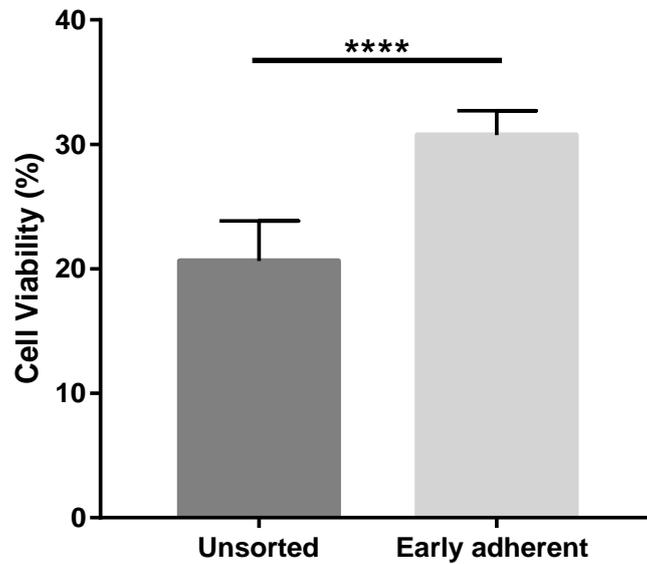


Figure 3.14: The chemoresistance of early adherent H357 cells after treatment with 15 μ M cisplatin compared to that of unsorted cells. The dark grey column represents the cell viability of unsorted cells phenotype, while the light grey column represents the cell viability of early adherent phenotype. The error bar represents SEM. P value < 0.0001.

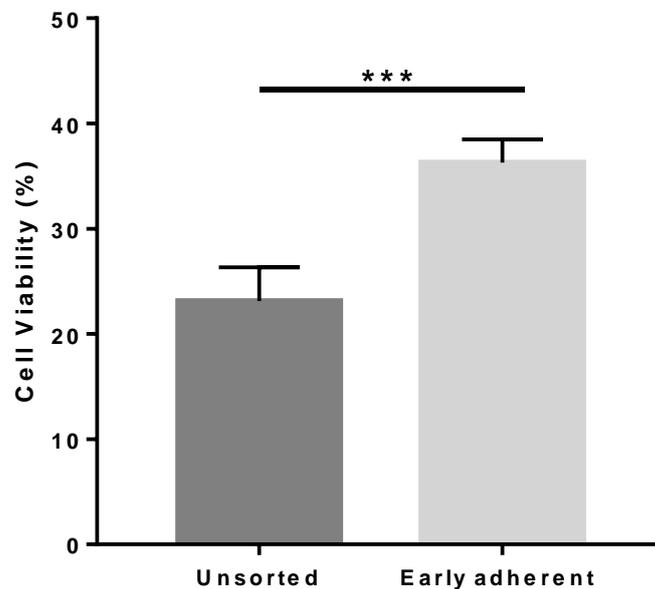


Figure 3.15: The chemoresistance of early adherent SCC4 cells after treatment with 15 μ M cisplatin compared to that of unsorted cells. The dark grey column represents the cell viability of unsorted cells phenotype, while the light grey column represents the cell viability of early adherent phenotype. The error bar represents SEM. P value = 0.0002.

3.4.7 Determination the adhesion of the chemoresistant cells

Adhesion assays were performed on the isolated chemoresistant cells from OSCC cell lines to identify their adhesion to fibronectin and compare it to that of unsorted cells.

The results demonstrate significantly higher adhesion of the isolated chemoresistant cells of both cell lines H357 (P value <0.0001) and SCC4 (P value = 0.0002) compared to their unsorted controls, (figures 3.16 and 3.17).

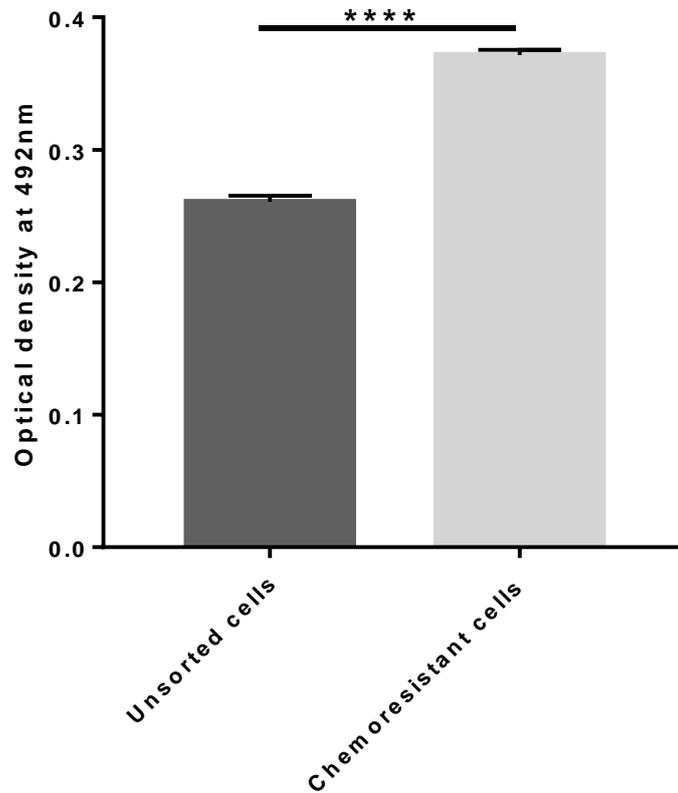


Figure 3.16: The adhesion of chemoresistant H357 cells and unsorted cells to the fibronectin. The light grey column represents the adhesion rate of the chemoresistant phenotype, while the dark grey column represents the adhesion rate of unsorted cells phenotype. The error bar represents SEM. P value < 0.0001.

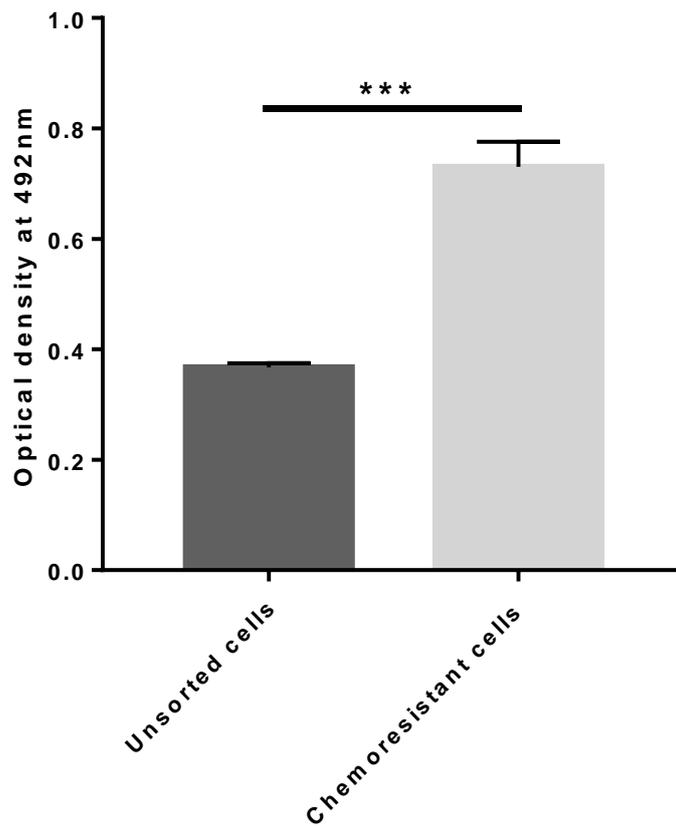


Figure 3.17: The adhesion of chemoresistant SCC4 cells and unsorted cells to the fibronectin. The light grey column represents the adhesion rate of the chemoresistant phenotype, while the dark grey column represents the adhesion rate of unsorted cells phenotype. The error bar represents SEM. P value = 0.0002.

3.5 Discussion

3.5.1 Assessment of the effects of time, ECM protein and oral cancer cell line on cell adhesion and isolation of early adherent cells.

Collagen I is one of the most common extracellular matrix molecules and is arranged as fibrils in the connective tissue of different body tissues particularly that are exposed to pressure, tensile or shear forces. It is formed by mesenchymal cells such as fibroblasts and myofibroblasts (Bosman and Stamenkovic, 2003). Whereas, fibronectin is a matrix glycoprotein occurs in cell matrix throughout body tissues (Kosmehl *et al.*, 1999) and has an important role in cell proliferation, adhesion and migration (Ioachim *et al.*, 2005). Both collagen I and fibronectin are considered as chemoattractants for oral cancer cell lines (Bitu *et al.*, 2012). They provide anchorage to oral cancer cells facilitating their growth, spread and subsequent migration and invasion (Radisky, 2005). Attachment of cells to ECM proteins such as collagens, fibronectin and laminin are mediated by integrin family of surface receptors. Integrins are heterodimers consisting of 2 transmembrane glycoproteins which are non-covalently associated called α and β subunits. There are many possible combinations of these subunits which define the substrate binding specificity. For example, $\alpha 2\beta 1$ (receptor of collagen), $\alpha 3\beta 1$ (receptor of laminin), $\alpha 5\beta 1$ (receptor of fibronectin) and $\alpha v\beta 6$ (receptor for tenascin and fibronectin) (Watt and Hertle, 1994). There are variations in integrin receptor expression with the highest levels confined to basal cell layer including stem cells and their levels are inversely related to cell differentiation. In addition, to their role in adhesion and migration of cells, integrins can regulate the differentiation of cells through signals transduction between the cells and extracellular microenvironment (Watt, 2002).

Our data showed a significant rapid adhesion of OSCC lines H357 and SCC4 to both collagen I and fibronectin compared to the adherence of controls. This might attributed to the high expression and increased functional activity of $\beta 1$ integrin receptors (Jones and Watt, 1993; Jones *et al.*, 1995). Furthermore, several studies found a strong correlation between cell adhesion to ECM proteins and the levels of $\beta 1$ integrins, i.e. significant reduction of $\beta 1$ integrins levels and/or down-regulating of functional activity of pre-existing $\beta 1$ integrins leads to a significant decrease in adhesiveness of cells to ECM proteins (Adams and Watt, 1989; Hotchin and Watt,

1992; Jones and Watt, 1993). Therefore, to confirm that rapid adherence of isolated early adherent cells was due to the high expression of $\alpha 5\beta 1$ integrin, we could use an antibody specifically block it and see this reduces and inhibits rapid adhesion of these cells.

Our results showed that adhesion of both cell lines (H357 and SCC4) to collagen I was significantly higher at all time points compared to fibronectin and reached a maximum after 30 minutes compare to fibronectin (section 3.4.1). This could be due to the fact that collagen receptors ($\alpha 2\beta 1$) are constitutive receptors and considered as the most abundant epithelial integrin receptors (Watt and Hertle, 1994) while fibronectin receptors ($\alpha 5\beta 1$ and $\alpha v\beta 6$) are induced in culture or in certain pathological condition such as upon wounding (Watt and Hertle, 1994; Breuss *et al.*, 1995; Zambruno *et al.*, 1995; Häkkinen *et al.*, 2000).

Therefore, the large number of rapidly adhering cells to collagen I could render it unsuitable for sorting of CSCs and this is consistent with studies by Kaur and Li (2000) who considered collagen I and IV inappropriate for isolation of keratinocyte stem cells from basal layer cells due to the expression of functionally active $\alpha 2\beta 1$ (collagen receptors) by most cells of this layer. As a result, we used fibronectin to isolate CSCs in order to obtain a more purified population of cells and this is in agreement with a number of other studies (Jones and Watt, 1993; Douthwaite *et al.*, 2003; Hall *et al.*, 2006). Moreover, David and her colleagues (2010) used differential adhesion to fibronectin to isolate adult stem cells from oral mucosa lamina propria.

Many studies predict that the percentage of stem cells is less than 10% of the total unfractionated keratinocytes (Withers, 1967; Potten and Morris, 1988; Bickenbach and Chism, 1998; Clausen and Potten, 1990; Jones *et al.*, 1995). This is in accordance with our study (section 3.4.1), in that we found the number of rapidly adhering cells (the isolated cells) using fibronectin from both oral cancer cell lines was less than 10% of the average number of seeded cells (H357= 4.4% and SCC4= 5.5%).

3.5.2 Effect of cell detachment solution on cell adhesion

Our results showed that there was no difference in the adhesion rate of oral cancer cell lines to ECM proteins when either trypsin/EDTA or Accutase cell detachment solutions were used. Therefore, we suggest that trypsin/EDTA does not disturb cell surface integrin expression. However, we used Accutase as detachment solutions for cells in flow cytometry experiments due to the fact that it is thought to preserve maximally cell surface proteins (Harper *et al.*, 2007).

3.5.3 Stability of the isolated phenotype (early adherent and chemoresistant phenotype)

In the current study, the stability of the phenotypes isolated using the two functional methods (adhesion and chemoresistance) from the both OSCC cell lines (H357 and SCC4) was investigated. The findings showed that the early adherent phenotypes from H357 and SCC4 exhibited significant higher levels of rapid adhesion to fibronectin after two days incubation in culture compared to unsorted cells, section (3.4.3). Furthermore, the results demonstrate that isolated chemoresistant populations from H357 and SCC4 cell lines expressed significantly less sensitivity to chemotherapeutic agent compared to untreated cells section (3.4.5).

To our knowledge there are no previous reports that have investigated the stability of the early adherent phenotype, whilst our findings on stability of the chemoresistant phenotype is in accordance with that reported by Kim, Roopra and Alexander, (2012) and Bertolini *et al.*, (2009). Kim and his colleagues (2012) found an increased mitotic index of CSC-like cells after a second administration of chemotherapeutic drug and that these cells were able to regenerate tumours after serial passages. Furthermore, Bertolini and his colleagues (2009) revealed that an isolated subpopulation of CD133 positive cells expressed high levels of stemness-associated genes and were resistant to cisplatin exposure, establishing a stable chemoresistant phenotype *in vitro*.

Several reports show that heterogeneous tumour populations have a tendency over time to maintain a fixed equilibrium of phenotypic proportions called a phenotypic equilibrium and that this has been shown *in vivo* as well as in cell lines *in vitro* (Gupta

et al., 2011; Iliopoulos *et al.*, 2011; Zapperi and La Porta, 2012). In addition, CSC have the ability to change their stem cell status through reversible fluctuations and this known as phenotypic plasticity (Chaffer *et al.*, 2011; Gupta *et al.*, 2011; Yang *et al.*, 2012). It has been reported that acquiring or losing stem-like characteristics by cancer cells in phenotype plasticity can be induced either by signals from the microenvironment or in response to anticancer therapy (Junttila and Sauvage, 2013; Lacina *et al.*, 2015).

Gupta and his colleagues (2011) reported that the phenotypic state of cancer cells is stable and the phenotypic equilibrium is maintained over time through inter-conversions between cancer cell phenotypic states under fixed conditions. Therefore, they proposed a model of cell-state dynamics (Markov chain) to explain the transition of phenotypes in breast cancer cell lines. Iliopoulos and his colleagues (2011) revealed in their study that the CSCs proportion in many cell lines is retained and stable over multiple generations due to switching between CSCs and cancer cells in a dynamic equilibrium.

Zapperi and La Porta, (2012) provided other explanations to the phenotypic equilibrium and stability of the CSC phenotype. They proposed two models: the phenotypic switching model and the imperfect CSC biomarker model. In the first model they suggest reversible transformation of cancer cells to a CSC state in order to retain the same discrimination between CSCs and cancer cells. On the other hand, the imperfect markers model was based on the inaccuracy of FACS sorting and suggests that the subpopulation with positive CSC markers is rich CSCs, while the subpopulation with negative CSC markers is low but not entirely devoid of CSCs. As a consequence, the CSCs (within the negative marker subpopulation) will re-establish the subpopulation with positive markers and subsequently drive tumourgenesis. Moreover, they found that there was no significance difference in the outcomes of the two models and both of them were in accordance with the Markov chain model reported by Gupta and his colleagues (2011).

Yang and his colleagues (2012) investigated two human cancer cell lines (colon and breast cell lines) in normal and irradiated conditions and found that there is an intrinsic homeostasis between CSCs and cancer cells. In addition, the stability of the CSC proportion was maintained due to the balance between the transition of cancer

cells to CSCs and the self-renewal, differentiation and asymmetric division of CSCs on the other side.

Liu and his colleagues (2013) examined the dynamic interaction between cancer cells and CSCs by developing a mathematical model to determine the proportion of CSCs. They found that the balance of CSC and cancer cell phenotypes and the maintenance of CSC proportion was controlled by negative feedback mechanisms of non-linear growth kinetics of CSCs.

3.5.4 Assessment of the sensitivity of oral cancer cell lines to cisplatin and isolation of chemoresistant cells.

Cisplatin is a well-known and potent anticancer agent, used in the treatment of a range of solid tumors including head and neck squamous cell carcinoma (Siddik, 2003). The cytotoxic action of cisplatin is mainly targeted to the DNA of cells (Eastman, 1983) leading to the formation of DNA adducts (Eastman, 1987). As a result of these adducts, many cellular functions controlled by DNA are affected such as suppression of DNA replication and RNA transcription as well as arresting of cell cycle (Zdraveski *et al.*, 2002; Vogelstein, Lane and Levine, 2000). In addition, apoptosis activating signals are mediated by DNA damage that result primarily from intrastrand crosslinks due to drug-DNA interactions (Eastman, 1987). Furthermore, the binding of damage recognition proteins (DRPs) which form part of the mismatch repair (MMR) complex with DNA physical distortions mediate the initiation of sequential events that extend from DNA adduct formation to the completion of apoptosis (Siddik, 2003).

As previously described, CSCs are more resistant to chemotherapeutic agents including cisplatin (section 1.2.4). Furthermore, because DNA is the main target of cisplatin, the chemoresistance of CSCs is attributed to the inhibition of signal propagation from DNA damage to apoptosis activation. Also there is increased repair of DNA adducts by several mechanisms such as: repair of DNA crosslinks by overexpression of topoisomerase II and activation of the NER pathway which is considered a crucial pathway in removal of platinum adducts (Siddik, 2003).

In our study, a wide range of cisplatin concentrations on both H357 and SCC4 cell lines were investigated from 0.5 μ M- 50 μ M (section 3.4.4) for 24 hours treatment and 5 days recovery time during which time cells continued to die. We selected an inhibitory concentration of 90% (IC₉₀) that yielded 10% of viable cells from the total number of treated cells which represents the chemoresistant cells (CSCs) and this in agreement with many studies that reported the percentage of CSCs in the total number of keratinocytes was equal or less than 10% (Withers, 1967; Potten and Morris, 1988; Bickenbach and Chism, 1998; Clausen and Potten, 1990; Jones *et al.*, 1995).

Recent studies have been conducted to isolate CSCs from different cancer cell lines using cisplatin and reported a variety of cisplatin concentrations and duration of treatment. For instance, Ma *et al.*, (2010) isolated CSCs from the SKOV3 ovarian cancer cell line using cisplatin at a concentration 40 μ M for 7 days, while Lopez-Ayllon *et al.*, (2014) reported in their study 2 concentrations of cisplatin to isolate CSCs from 2 different lung cancer cell lines H460 and A549 which were 1.7 μ M and 8.3 μ M respectively for 72 hours. However, our findings showed that the appropriate concentrations of cisplatin for isolation of CSCs from oral cancer cell lines were 15 μ M for the H357 cell line and 17.5 μ M for the SCC4 cell line for 24 hours. The wide variation of cisplatin concentrations and duration of treatment was attributed to the difference in cisplatin sensitivity and drug inhibitory responses between cancer cell lines (Carmichaell *et al.*, 1988; Barr *et al.*, 2013). The critical parameters in the isolation of CSCs using a chemoresistance assay are the concentration of chemotherapeutic agent, duration of treatment and the period of time during which the isolated cells stay viable after isolation (Cole *et al.*, 2014). Therefore, in this study we considered these parameters carefully in the assessment of the required concentration of cisplatin, period of treatment and viability of isolated cells during the recovery period.

Furthermore, the main limitation we faced in this experiment was the slow and continuous dying of cells during the recovery period that affected the final percentage of viable cells. Therefore, to overcome this problem we decided to reduce the time of treatment and selected the appropriate concentration of the drug as well as leaving the cells for 5 days for recovery in order to obtain 10% of viable cells. Similar issues were faced by Cole and his colleagues (2014) when they used the same protocol of Ma *et al.*, (2010) for isolation of CSCs from the SKOV3 ovarian cancer cell line.

Modifications of the protocol were made by reducing the cisplatin concentration from 40 μ M for 7 days to 20 μ M for 72 hours.

3.5.5 Determination the chemoresistance of early adherent cells and the early adhesion of the chemoresistant cells

These experiments were performed to investigate if there is a correlation between the phenotypes of cell populations isolated using the two different functional methods. This was considered a novel approach because almost all previous reported studies focused on isolation of CSCs either by FACS analysis or single functional assay and then further characterized by more functional assays.

Our findings showed that early adherent cells of both cancer cell lines (H357 and SCC4) expressed significantly higher levels of viability after cisplatin treatment in comparison to unsorted cells (section 3.4.6). This finding is in agreement with that reported by several studies that found enhanced cisplatin chemoresistance in cancer cells with increased adhesion to fibronectin (Sethi *et al.*, 1999; Matter and Ruoslahti, 2001; Nakahara *et al.*, 2003; Hartmann *et al.*, 2005). Several mechanisms have been suggested to explain this phenomenon. Sethi *et al.*, (1999) found that cancer cell adhesion to fibronectin activates $\alpha 5\beta 1$ integrin to mediate survival signals that trigger activation of tyrosine kinases that inhibit apoptosis induced by chemotherapy. Matter and Rouslahti, (2001) showed in their study that adhesion to fibronectin modulates chemoresistance by activation of survival signal pathway resulting in overexpression of the anti-apoptotic bcl-2 protein. The pathway mediated by $\alpha 5\beta 1$ integrin activates focal adhesion kinase (FAK) which in turn activates Ras and the PI3K-AKT pathway which enhances transcription of the bcl-2 protein. Nakahara and his colleagues (2003) revealed that adhesion to fibronectin mediated by a $\alpha 5\beta 1$ integrin in a head and neck squamous cell line activates phosphorylation of FAK that initiates a survival signaling pathway leading to increased chemoresistance to cisplatin. Furthermore, they found that treating cells with a neutralizing antibody to $\alpha 5\beta 1$ integrin leads to a complete abolishment of cisplatin-resistance rendering cells highly sensitive to cisplatin chemotherapy.

Our results revealed that the isolated chemoresistant cells of H357 and SCC4 cell lines demonstrated increased levels of rapid adherence to fibronectin compared to unsorted cells (section 3.4.7). This is in accordance with the study of Vallo *et al.*, (2017) who showed up-regulating levels of $\beta 1$ integrin expression in cisplatin-resistant urethral cancer cell lines and functional suppression of $\beta 1$ integrin leads to a significant reduction in the adhesion of these cells compared to untreated control cells.

3.6 Conclusions

We can conclude that cell adhesion is higher to collagen I than to fibronectin and maximal cell adhesion occurred after 30 minutes. Rapid adhesion to fibronectin at 10 minutes and chemoresistance to 15 μ M cisplatin isolate less than 10% of the average total number of the seeded cells for both oral cancer cell lines (H357 and SCC4). In addition, the increased chemoresistance of early adherent cells and the increased adherence of cisplatin-resistant cells suggest that these two methods are isolating phenotypically overlapping populations of CSCs. The isolated phenotype (early adherent and/or chemoresistant) was stable at least 48 hours after isolation.

CHAPTER 4
CHARACTERISATION OF
ISOLATED CELLS

4.1 Introduction

Characterisation of the biological and molecular properties of the cancer stem cells isolated in chapter (3) was considered a crucial step to verify the ‘stemness’ of the population in order to study their characteristics accurately with the aim of subsequent specific targeting and eradicating of these cells (Ailles and Weissman., 2007).

It has been reported that CSCs have distinctive characteristics discriminating them from other cancer cells within tumor which are: they form a minor population of cells, they are capable of self-renewal and differentiation to multiple non-tumourigenic cell lineages and they express of specific surface cell markers according to type of cancer (Clarke *et al.*, 2006; Dalerba *et al.*, 2007) as well as they are tumourigenic and resist to conventional anticancer therapy (Reya *et al.*, 2001). In addition, there is a distinctive gene expression profile reflecting the biology of these CSCs that drives the progression of tumour (Sgroi *et al.*, 1999; Leethanakul *et al.*, 2001).

In general, two approaches were used to characterise CSCs: investigation of specific protein expression and functional assays (Dobbin and Landen, 2014).

In the current study, we characterised the sorted cells from the two functional methods (adhesion and chemoresistance) using FACS analysis and gene-expression profile of the three common head and neck squamous cell carcinoma cancer stem markers (CD24, CD44 and CD29). In addition, proliferation and colony forming assays were performed as functional measures of ‘stemness’ as well as examination of capability of the isolated cells to secrete extracellular vesicles. This was performed as EVs have roles in communication between various cells in tumour microenvironment, as well as mediation of different signalling pathways that enhance proliferation, survival and anticancer in tumour cells (Hannafon and Ding, 2013).

4.2 The aims of the chapter

1. To investigate the expression of stem cell surface markers in the isolated cells.
2. To investigate the expression of stem cell associated genes in the isolated cells.
3. To compare the proliferation capacity of the isolated cells to that of unsorted cells.
4. To investigate the clonogenic potential of the isolated cells.
5. To investigate the capability of isolated cells to secrete extracellular vesicles in comparison to that of unsorted cells.

4.3 Methods

The following methods were used in this chapter:

- Cell culture (section 2.2)
- Isolation of early adherent cells by adhesion assay (section 2.3)
- Isolation of chemoresistant cells by chemoresistance assay (section 2.5)
- Flow cytometry for stem cell surface markers expression (section 2.11)
- RNA extraction from isolated and unsorted cells (section 2.12)
- cDNA synthesis (section 2.13)
- Real time –Polymerase chain reaction (qPCR) (section 2.14)
- Proliferation assay (section 2.9)
- Colony forming assay (section 2.10)
- Determination of extracellular vesicles produced by early adherent and chemoresistant cells (section 2.18)

4.4 Results

4.4.1 Stem cell surface markers analysis by flow cytometry

In this experiment, the expression of three well-known head and neck squamous cell carcinoma specific stem cell surface markers (CD24, CD44 and CD29) were investigated using FACS analysis in the isolated cells from both oral cancer cell lines using rapid adhesion and chemoresistance and the levels compared to that of unsorted cells.

The results showed that isolated early adherent cells of the both cell lines H357 and SCC4 expressed significantly higher levels of all the examined stem cell surface markers (CD24, CD44 and CD29) compared to the unsorted cells, as illustrated in figures 4.1 and 4.2 respectively.

The enhanced expression of CD29 marker in particular by the early adherent cells of both examined oral cancer cell lines compared to unsorted cells was expected because $\beta 1$ integrins are the main receptors that bind cells to the ECM proteins. Moreover, this finding could explain and support our results in previous (chapter 3), that showed early adherent cells rapidly attached to fibronectin compared to unsorted cells.

The chemoresistant cells isolated from H357 and SCC4 oral cancer cell lines also exhibited significantly increased levels of CD24, CD44 and CD29 in comparison to unsorted cells, as shown in figures 4.3 and 4.4.

However, our data showed that the biggest difference in expression of stem cell markers between early adherent H357 cells and unsorted cells was in CD44 expression. Whereas, CD24 was expressed by both early adherent SCC4 cells and chemoresistant H357 with biggest differences compared to unsorted cells. Finally, CD29 represents the stem cell marker with biggest difference in expression between chemoresistant SCC4 and unsorted cells.

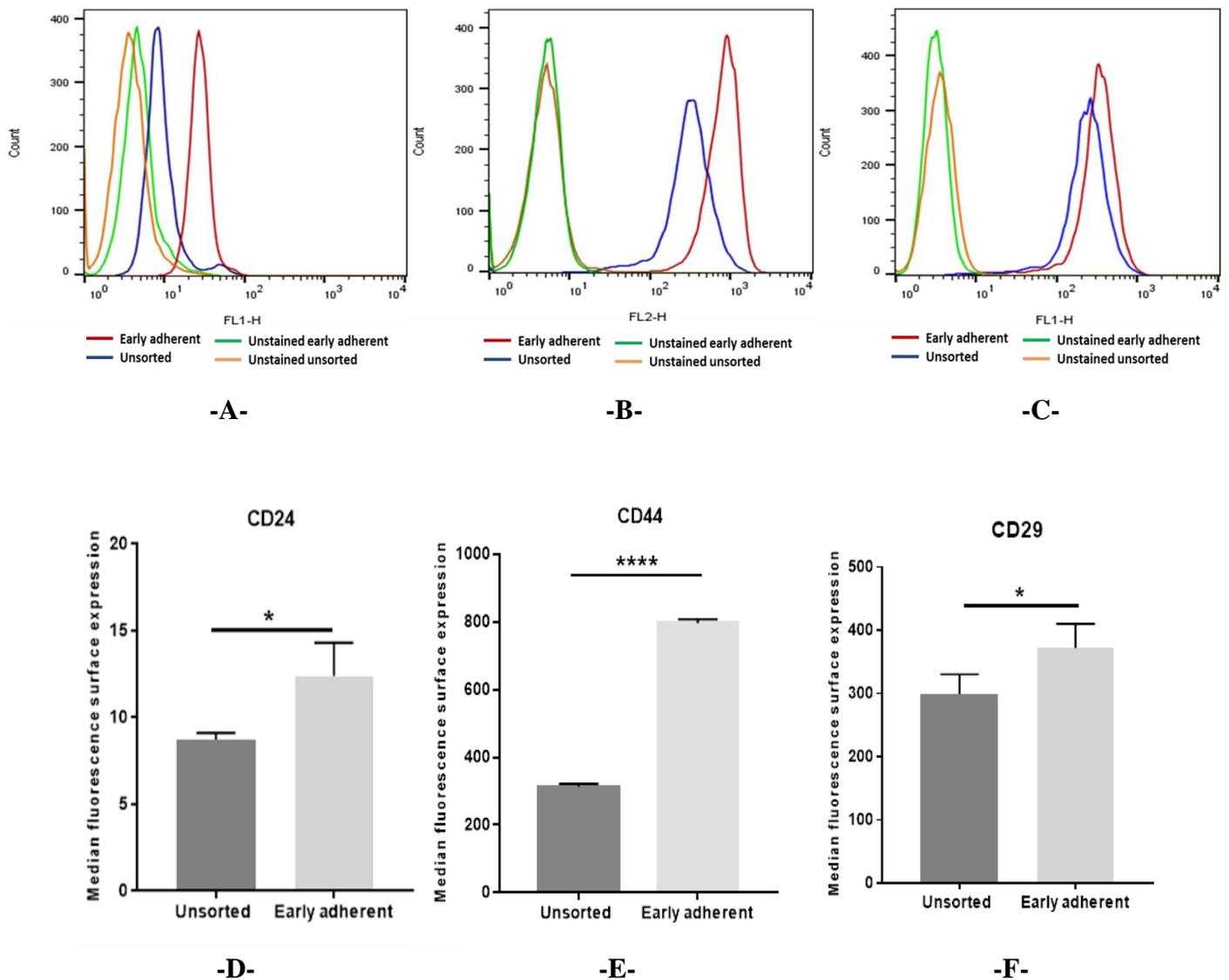


Figure 4.1: Expression of stem cell surface markers by early adherent and unsorted H357 cells. (A, B, C) Representative histograms of the median fluorescence value were plotted against the number of CD24, CD44 and CD29 positive cells; respectively, early adherent cells (red), unsorted cells (blue) early adherent unstained –negative control- (green) and unsorted unstained– negative control- (brown). The graphs represent one of at least 3 independent experiments. (D, E, F) Histogram bars represent means \pm SD of three independent experiments of expression of CD24, CD44 and CD29 respectively by early adherent cells (light grey) and unsorted cells (dark grey). *, **** Indicate statistically significant differences at (P < 0.05) and (P < 0.0001) respectively.

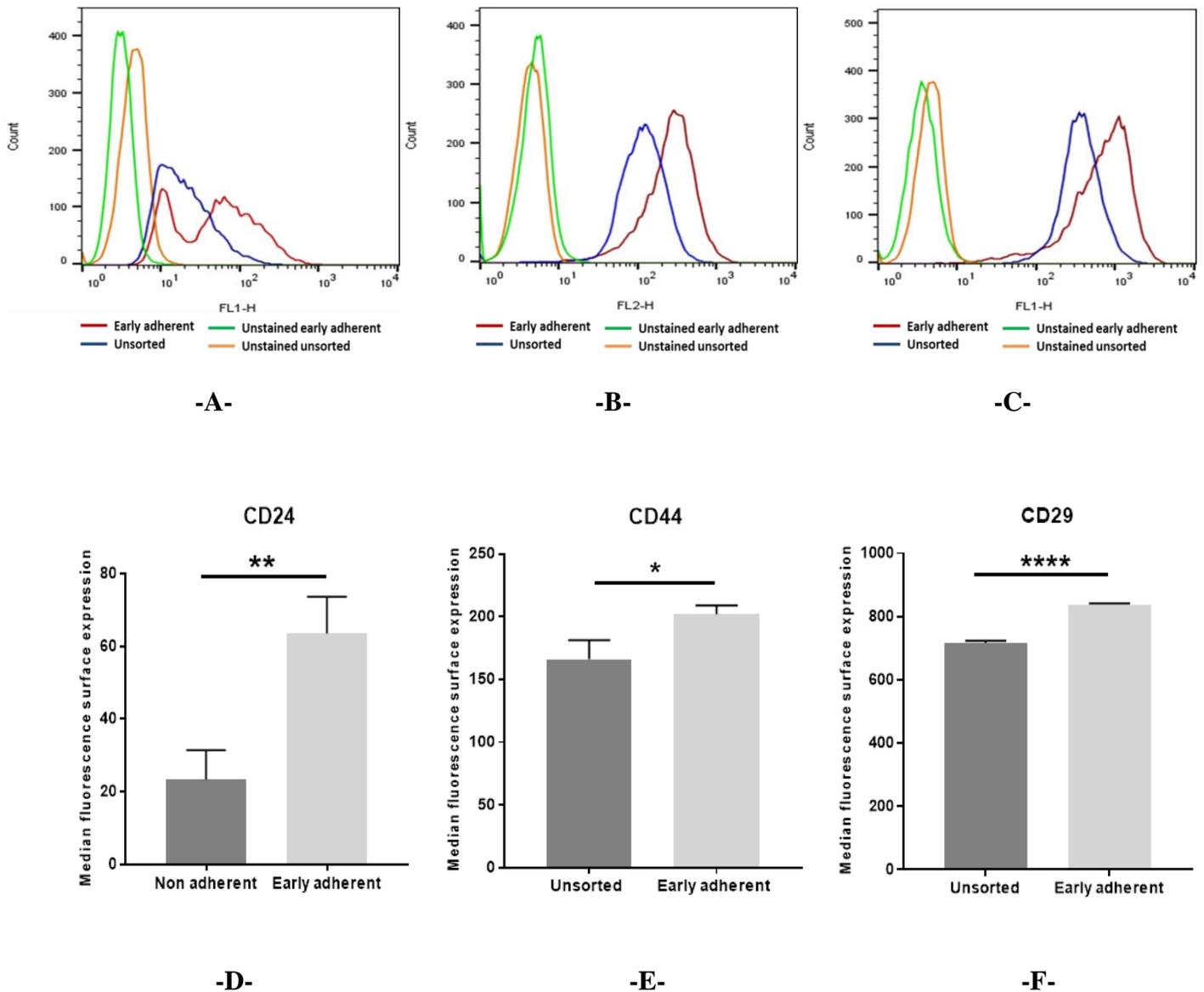


Figure 4.2: Expression of stem cell surface markers by early adherent and unsorted SCC4 cells. (A, B, C) Representative histograms of the median fluorescence value were plotted against the number of CD24, CD44 and CD29 positive cells; respectively, early adherent cells (red), unsorted cells (blue) early adherent unstained –negative control- (green) and unsorted unstained –negative control- (brown). The graphs represent one of at least 3 independent experiments. (D, E, F) Histogram bars represent means \pm SD of three independent experiments of expression of CD24, CD44 and CD29 respectively by early adherent cells (light grey) and unsorted cells (dark grey). *, **, **** Indicate statistically significant differences at ($P < 0.05$), ($P < 0.001$) and ($P < 0.0001$) respectively.

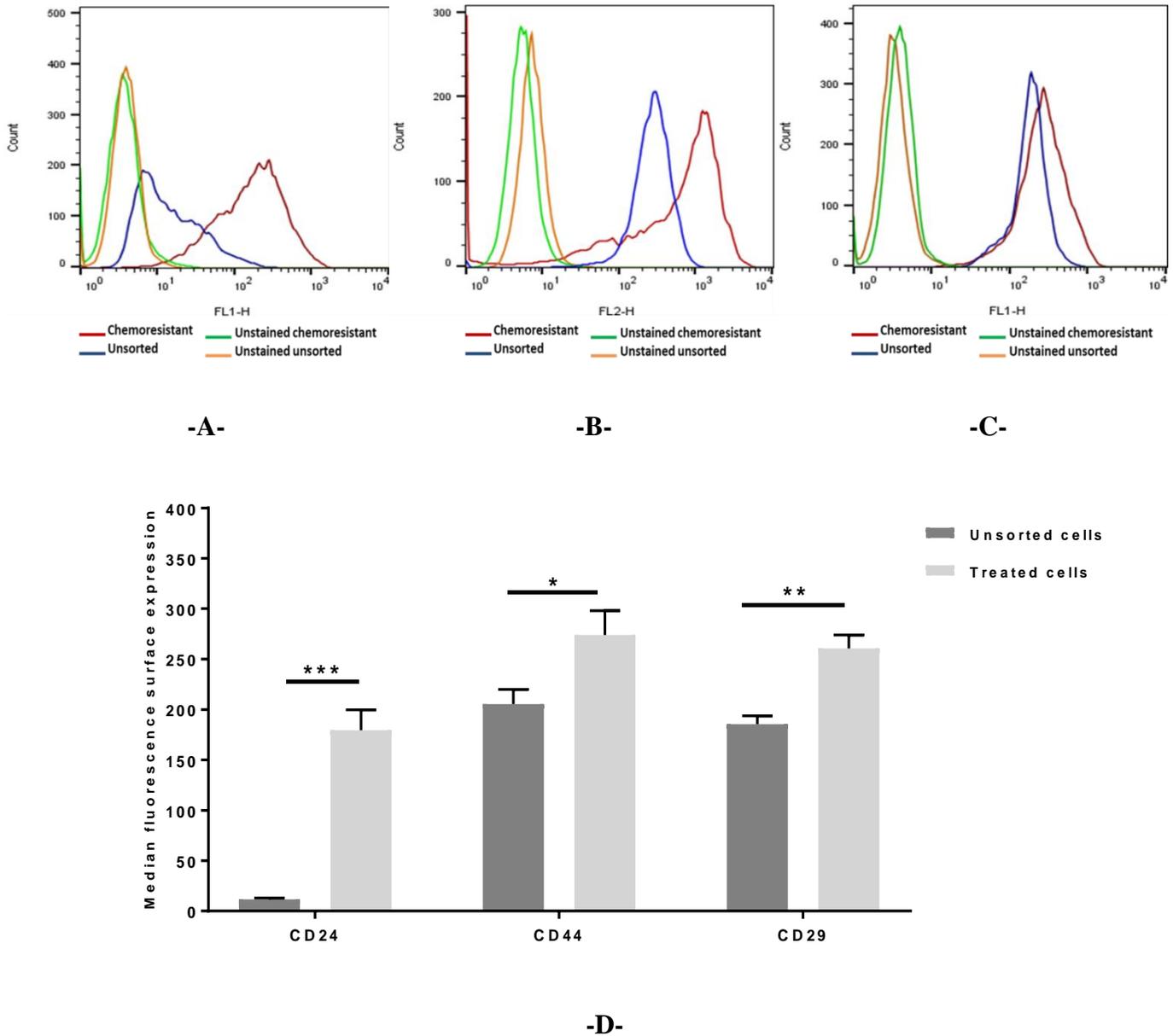
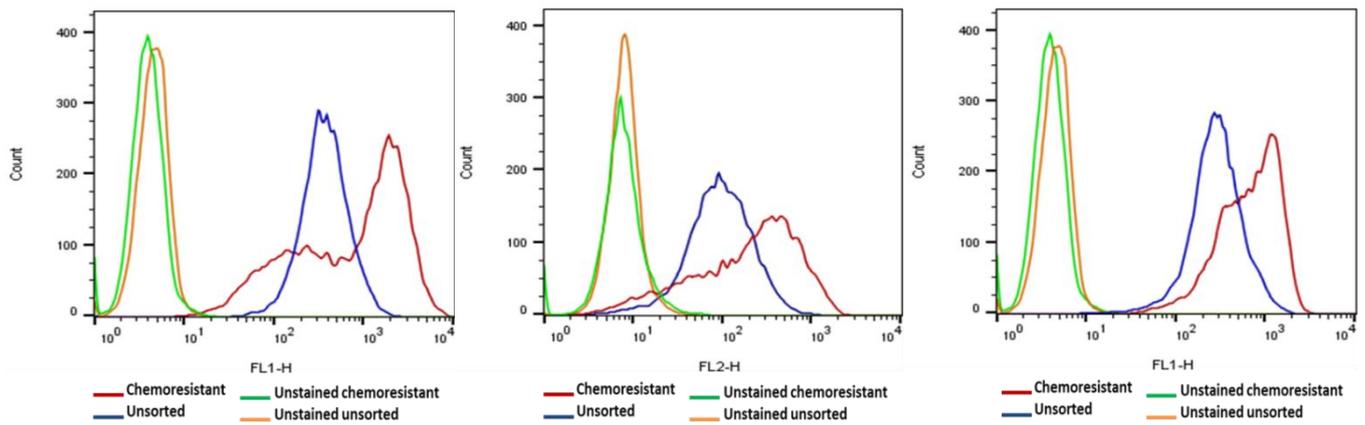


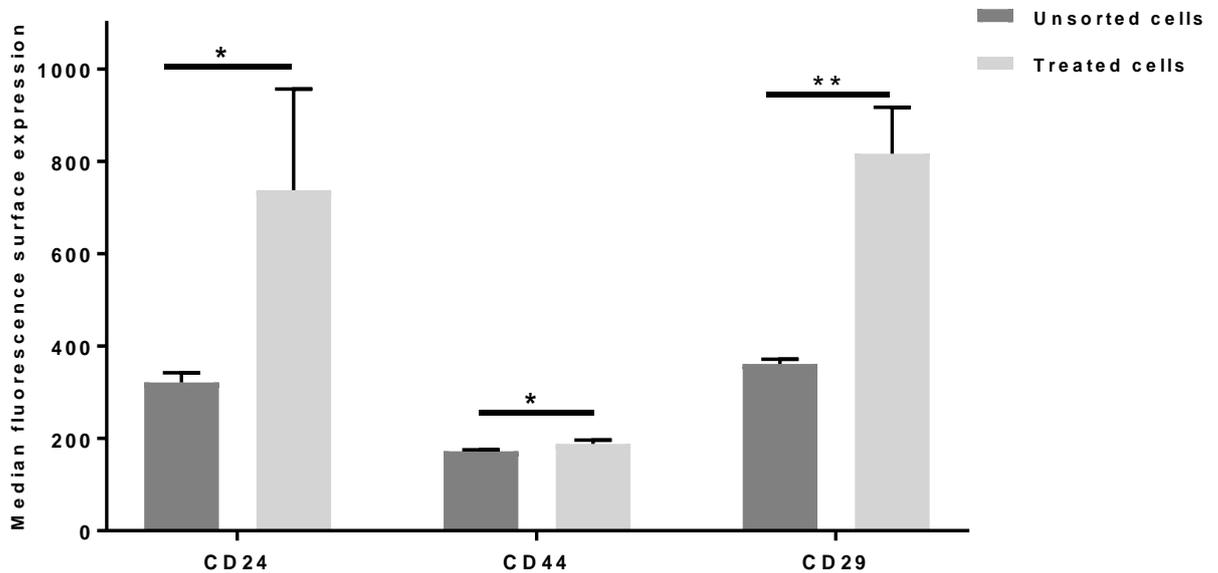
Figure 4.3: Expression of stem cell surface markers by chemoresistant and unsorted H357 cells. (A, B, C) Representative histograms of the median fluorescence value were plotted against the number of CD24, CD44 and CD29 positive cells; respectively, chemoresistant cells (red), unsorted cells (blue) unstained chemoresistant –negative control- (green) and unstained unsorted–negative control- (brown). The graphs represent one of at least 3 independent experiments. (D) Histogram bars represent means \pm SD of three independent experiments of expression of CD24, CD44 and CD29 respectively by early adherent cells (light grey) and unsorted cells (dark grey). *, **, *** Indicate statistically significant differences at ($P < 0.05$), ($P < 0.001$) and ($P < 0.0001$) respectively.



-A-

-B-

-C-



-D-

Figure 4.4: Expression of stem cell surface markers by chemoresistant and unsorted SCC4 cells. (A, B, C) Representative histograms of the median fluorescence value were plotted against the number of CD24, CD44 and CD29 positive cells; respectively, chemoresistant cells (red), unsorted cells (blue) unstained chemoresistant –negative control- (green) and unstained unsorted–negative control- (brown). The graphs represent one of at least 3 independent experiments. (D) Histogram bars represent means \pm SD of three independent experiments of expression of CD24, CD44 and CD29 respectively by early adherent cells (light grey) and unsorted cells (dark grey). *, ** Indicate statistically significant differences at ($P < 0.05$) and ($P < 0.001$) respectively.

4.4.2 Genetic characterisation of isolated CSCs

The RNA expression of stem cell associated genes was determined in isolated CSCs (early adherent and chemoresistant) and compared with that of unsorted cells using quantitative real time-PCR (qPCR) analysis. Three of commonly used head and neck squamous cell carcinoma ‘stemness’ related genes were selected to be investigated in this experiment which were CD24, CD44 and CD29 (Han *et al.*, 2009; Ling *et al.*, 2014).

Our findings of qPCR analysis revealed that all examined genes (CD24, CD44 and CD29) were significantly up-regulated in the isolated early adherent cells from both cancer cell lines (H357 and SCC4) compared to that in unsorted cells. The fold increase of the early adherent cells of the H357 cell line in comparison with that of unsorted cells were as follows: 6 fold for CD24, 4 fold for CD44 and 2 fold for CD29 respectively (figure 4.5). Whereas, the elevated fold change in early adherent cells of SCC4 cell line were: 2 fold for CD24 and 1.5 fold for both CD44 and CD29 compared to that in unsorted cells (figure 4.6).

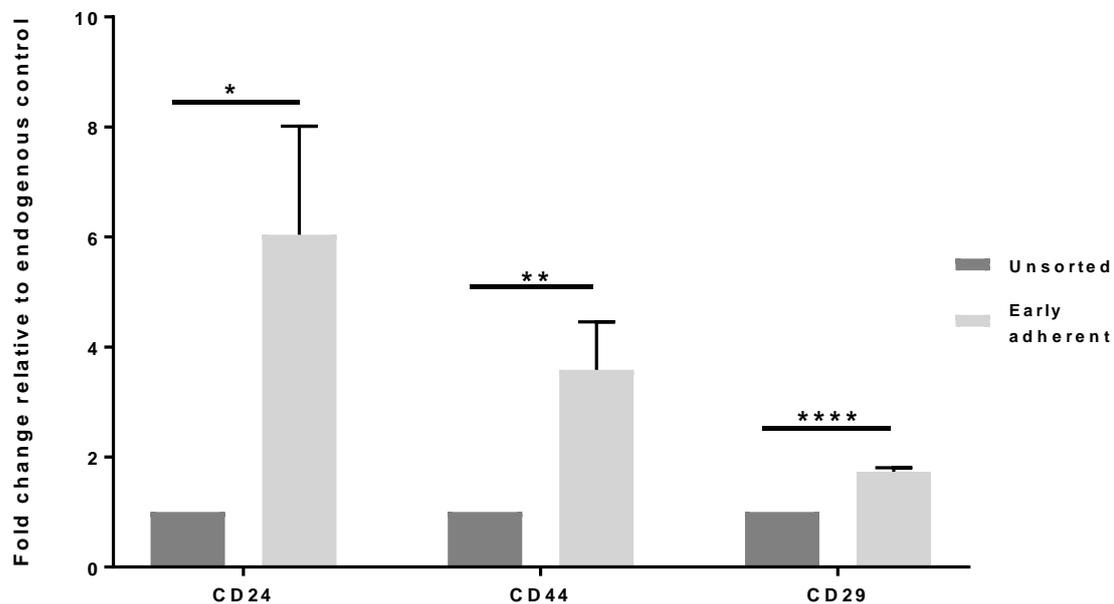


Figure 4.5: The mean fold change in CD24, CD44 and CD29 stem associated gene expression by the early adherent cells (light grey) of H357 cell line relative to the unsorted control (dark grey), \pm SD of at least 3 independent experiments performed in triplicate. *, **, **** Indicate statistically significant differences at ($P < 0.05$), ($P < 0.001$) and ($P < 0.0001$) respectively.

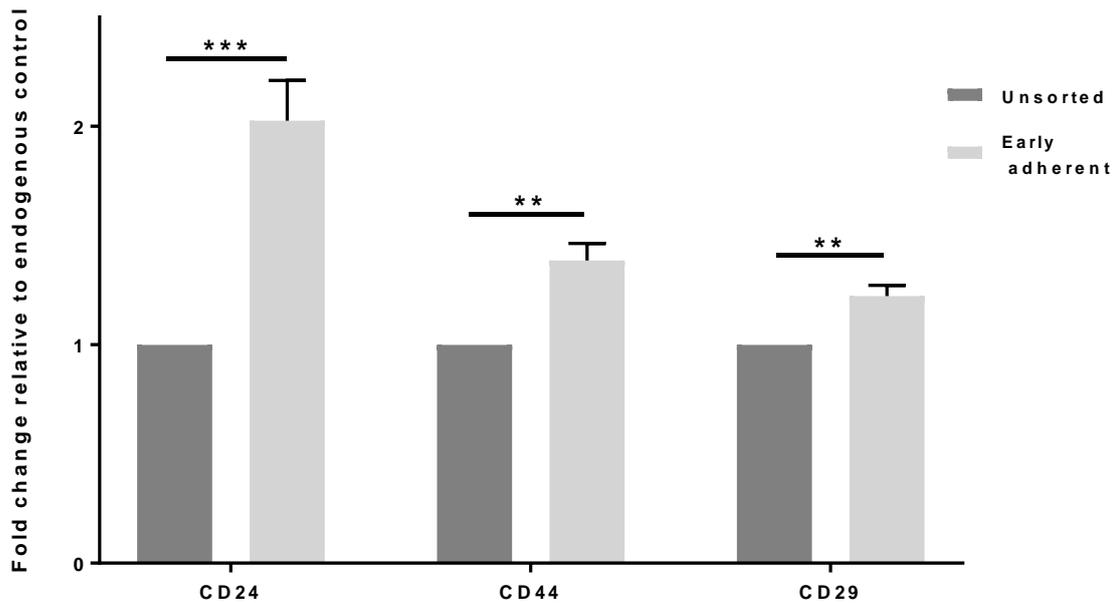


Figure 4.6: The mean fold change in CD24, CD44 and CD29 stem cell associated gene expression by the early adherent cells (light grey) of SCC4 cell line relative to the unsorted control (dark grey), \pm SD of at least 3 independent experiments performed in triplicate. **, *** Indicate statistically significant differences at ($P < 0.001$) and ($P < 0.0001$) respectively.

The isolated chemoresistant cells from H357 showed significant up-regulation of the investigated genes as follows: 55 fold for CD24, 12 fold for CD44 and 2 fold for CD29 expression compared to unsorted control as illustrated in the figures 4.7 A, B and C respectively. At the same time, qPCR analysis of the chemoresistant and unsorted cells of SCC4 cell line delineated that chemoresistant cells expressed 11 fold higher levels of CD24 and CD44 genes as well as 3 fold higher CD29 compared to unsorted (figure 4.8).

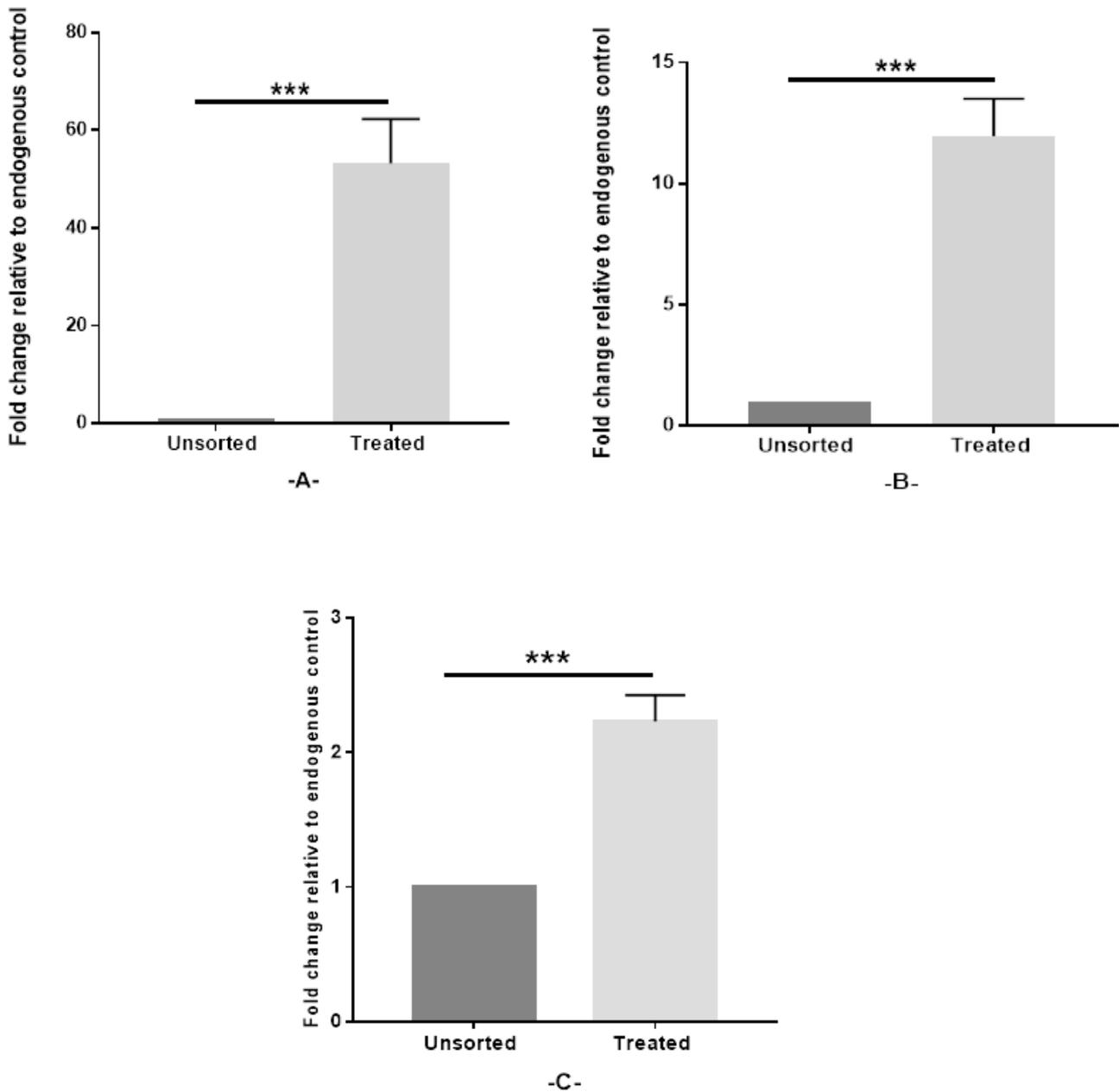


Figure 4.7: The mean fold change in (A) CD24, (B) CD44 and (C) CD29 stem cell associated gene expression by the chemoresistant cells (light grey) of H357 cell line relative to the unsorted control (dark grey), \pm SD of at least 3 independent experiments performed in triplicate. *** Indicate statistically significant differences (P<0.0001).

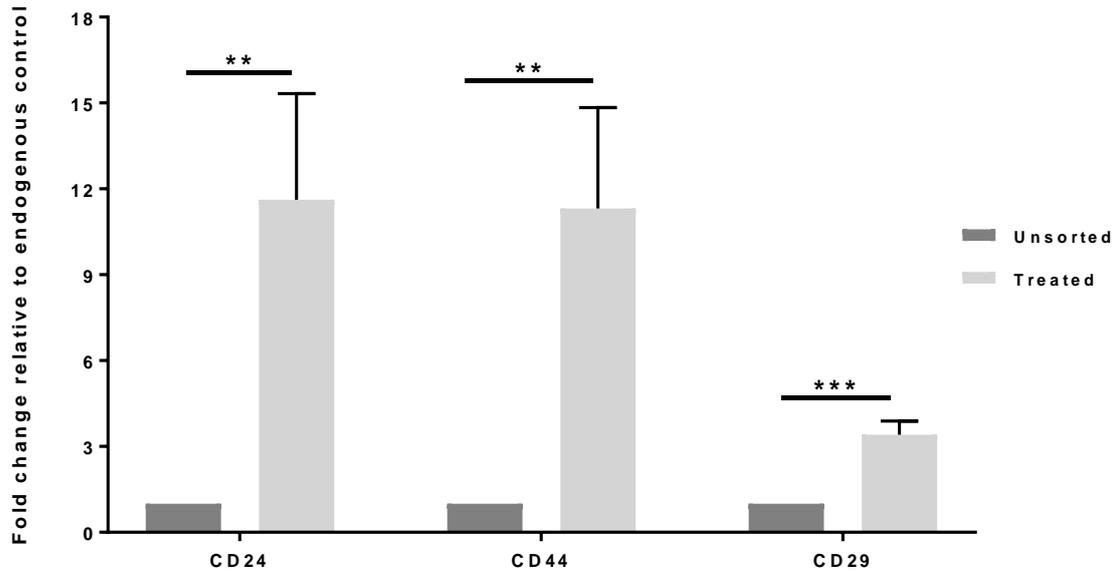


Figure 4.8: The mean fold change in CD24, CD44 and CD29 stem cell associated gene expression by the chemoresistant cells (light grey) of SCC4 cell line relative to the unsorted control (dark grey), \pm SD of at least 3 independent experiments performed in triplicate. **, *** Indicate statistically significant differences at ($P < 0.001$) and ($P < 0.0001$) respectively.

4.4.3 Proliferation assay

This assay was performed as it has been previously shown that stem cells are slow cycling and have a proliferation rate which is lower than normal cells (Roesch *et al.*, 2010; Pece *et al.*, 2010). Therefore, growth curves were plotted on the basis of proliferation rate of both isolated and unsorted cells over a period of 96 hours after isolation.

The study identified a significant reduction in the proliferation rate of cells isolated by rapid adherence of both H357 and SCC4 cell lines over time in comparison to the highly elevated growth rate of unsorted cells, figure 4.9 and 4.10.

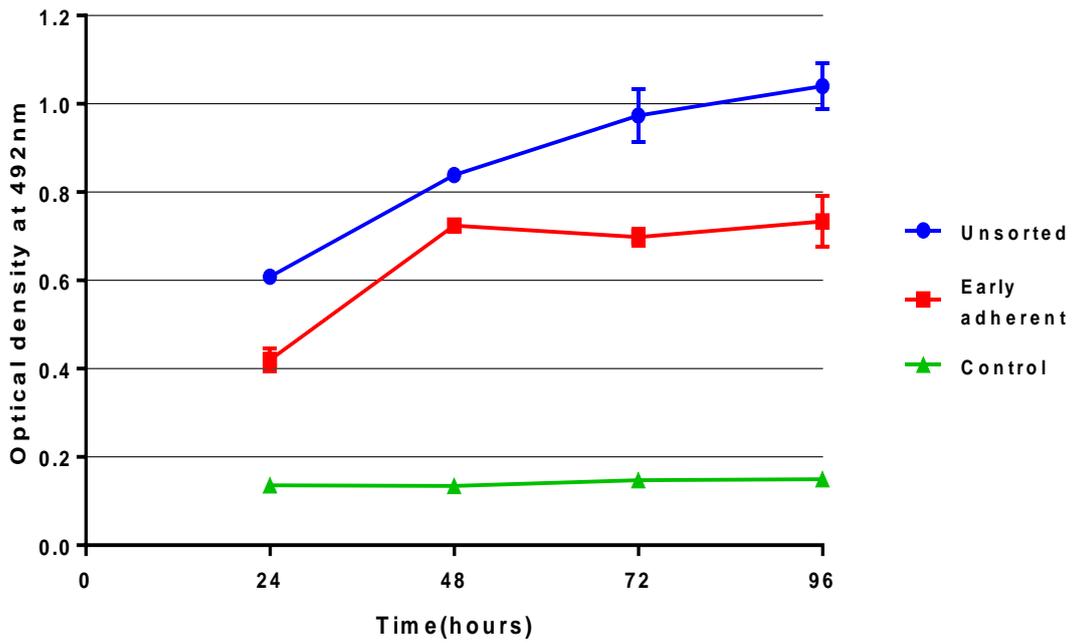


Figure 4.9: The growth curves of H357 cells showing the proliferation of unsorted cells (blue line), early adherent cells (red line) and control (green line) at different time points. All the assays were performed in triplicate and repeated three times. The error bar represents SEM. (p-value<0.0001).

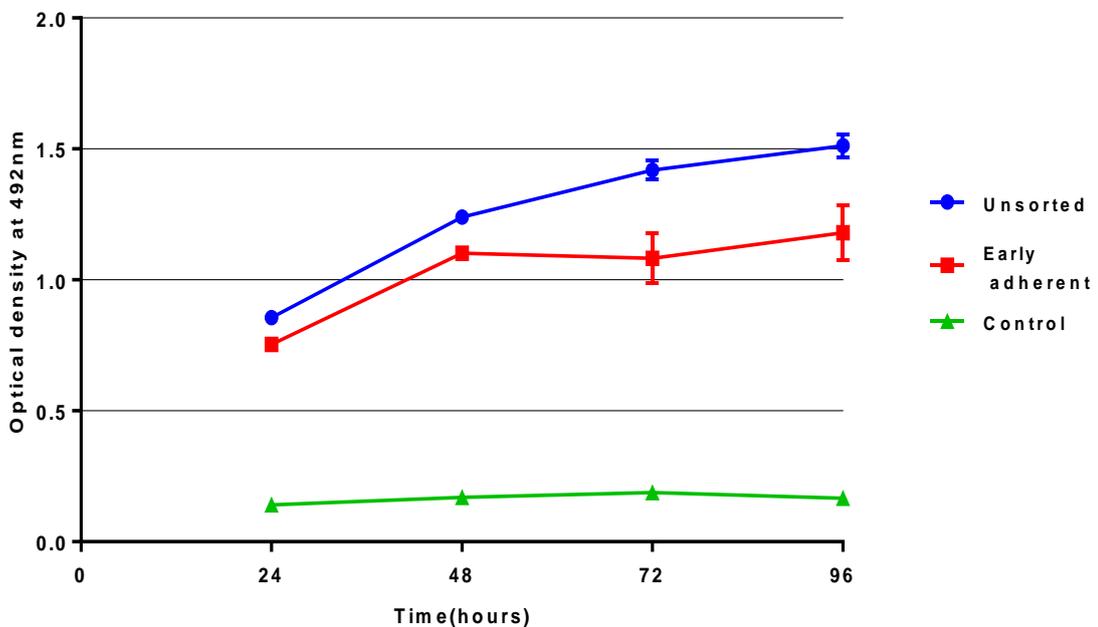


Figure 4.10 : The growth curves of SCC4 cells showing the proliferation of unsorted cells (blue line), early adherent cells (red line) and control (green line) at different time points. All the assays were performed in triplicate and repeated three times. The error bar represents SEM. (p-value<0.0001).

Meanwhile, the isolated chemoresistant cells from both OSCC cell lines (H357 and SCC4) exhibited a minimal growth rate while the unsorted cells showed highly increasing proliferation rate over time. There was a significant difference between the growth rate of chemoresistant and unsorted cells in the both cancer cell lines, figures 4.11 and 4.12.

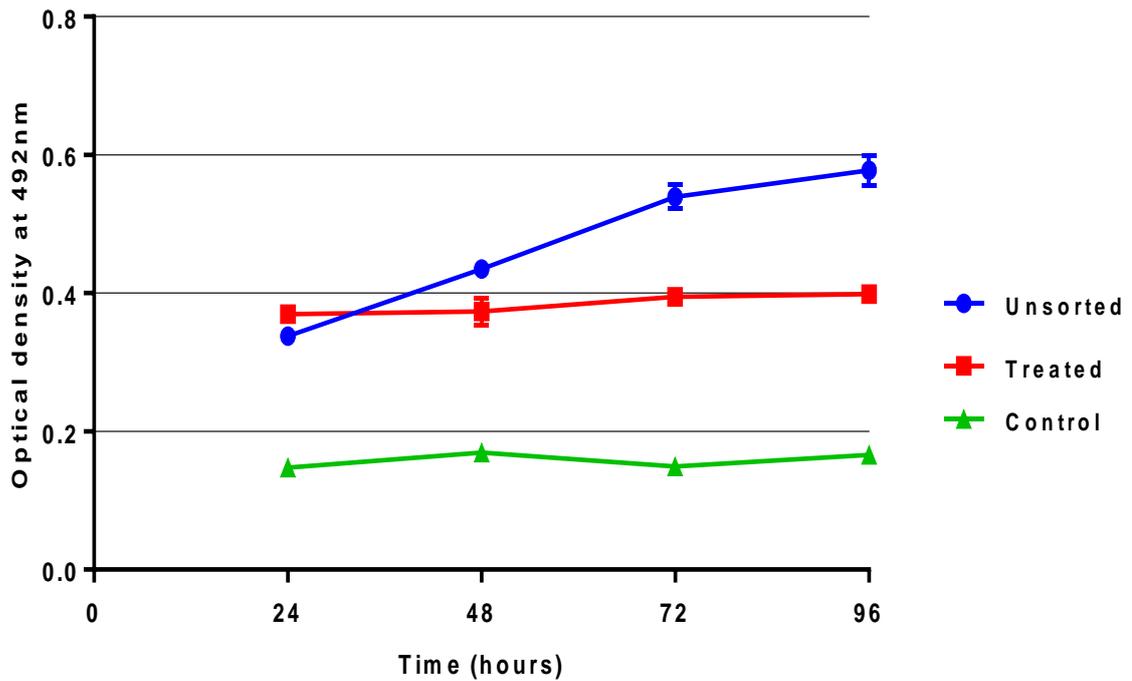


Figure 4.11: The growth curves of H357 cells showing the proliferation of unsorted cells (blue line), chemoresistant cells (red line) and control (green line) at different time points. All the assays were performed in triplicate and repeated three times. The error bar represents SEM. (p-value<0.05).

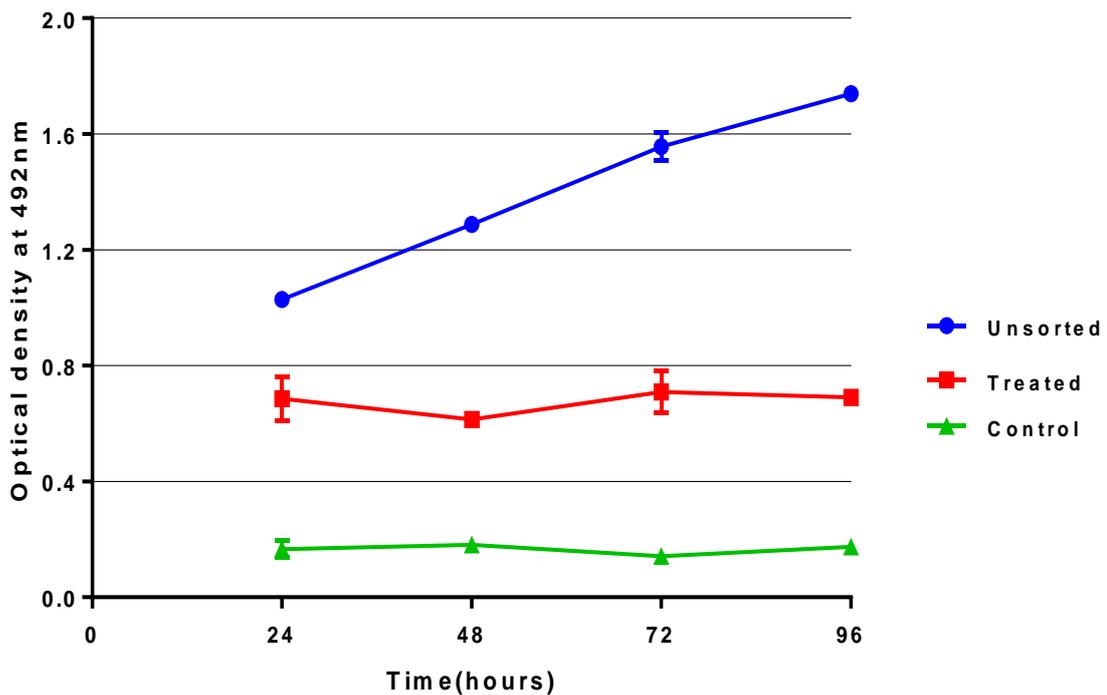


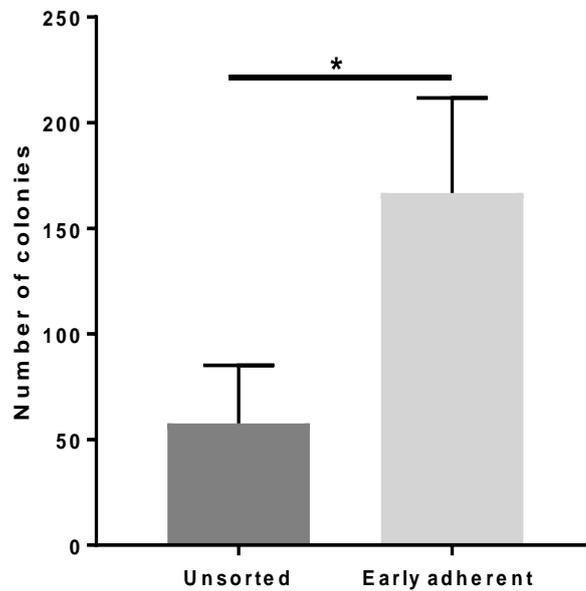
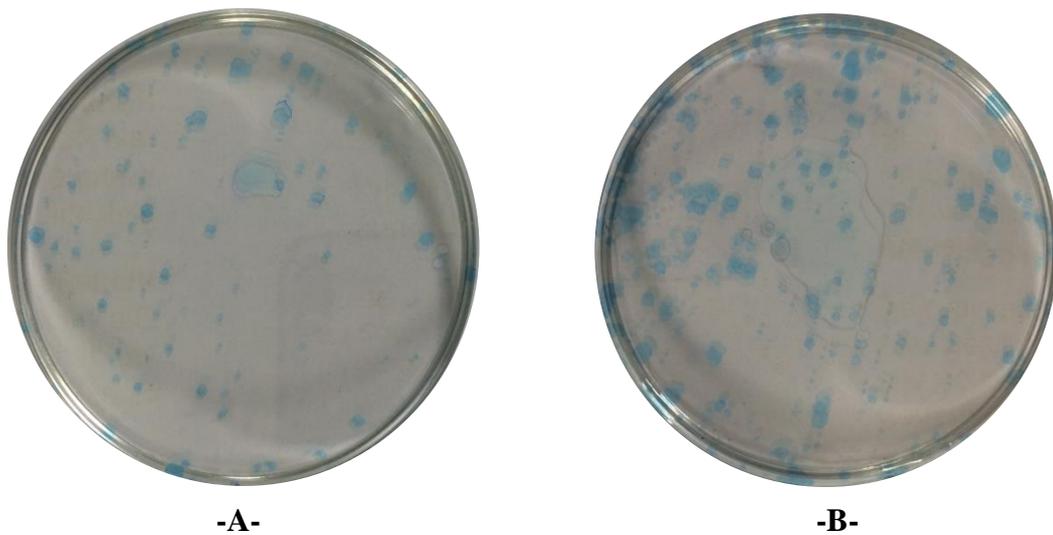
Figure 4.12: The growth curves of SCC4 cells showing the proliferation of unsorted cells (blue line), chemoresistant cells (red line) and control (green line) at different time points. All the assays were performed in triplicate and repeated three times. The error bar represents SEM. (p -value <0.0001).

4.4.4 Colony forming assay

This assay was performed in order to assess the clonogenicity of the early adherent and unsorted cells by seeding at a low density and allowing them to grow for 14 days after isolation.

The early adherent cells of H357 and SCC4 oral cancer cell lines demonstrated significantly higher clonogenic capacity in their ability to form a large number of colonies compared to unsorted cells, figures 4.13 and 4.14.

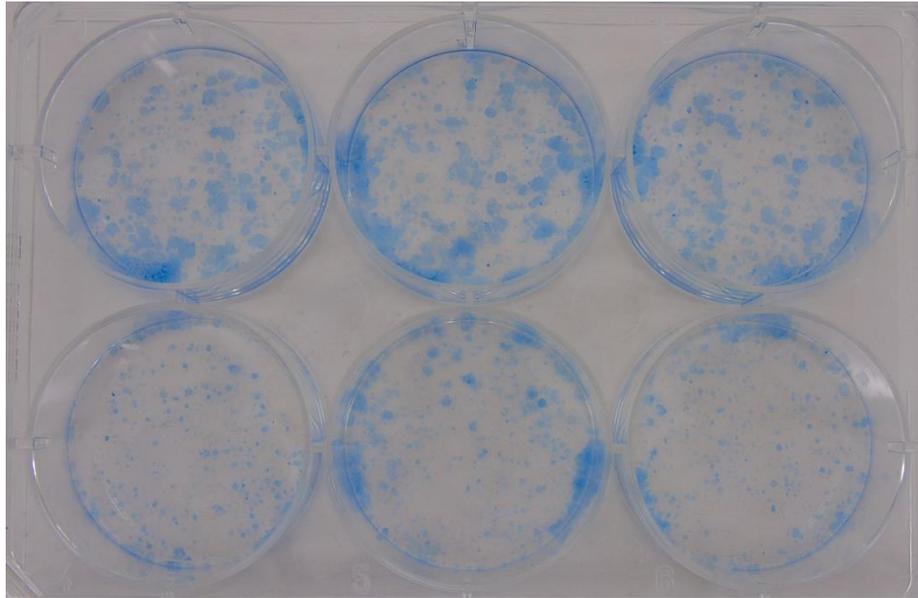
On the other hand, this assay was repeated 3 times on chemoresistant cells of both cell lines H357 and SCC4 and showed no colonies were formed after 14 days.



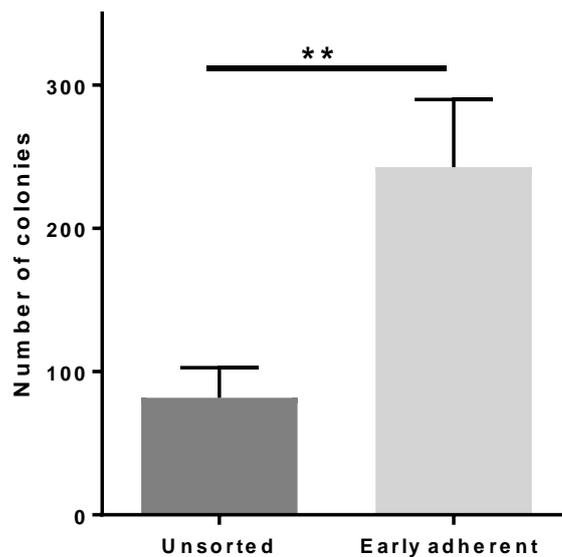
-C-

Figure 4.13: Colony forming ability of early adherent and unsorted H357 cells.

(A) Photograph of a well showing the colonies formed by unsorted cells after seeding of 1000 cells and incubation for 14 days. (B) Photograph of a well showing the colonies formed by early adherent cells after seeding of 1000 cells and incubation for 14 days. (C) Histogram showing the number of colonies formed by unsorted (dark grey column) and early adherent H357 cells (light grey column) after 14 days. All the assays were performed in triplicate and repeated three times. The error bar represents SEM. (p-value<0.05).



-A-



-B-

Figure 4.14: Colony forming ability of early adherent and unsorted SCC4 cells.

(A) Photograph of a 6 well plate showing the colonies formed by early adherent cells (upper row wells) and unsorted cells (lower row wells), after seeding of 1000 cells and incubation for 14 days. (B) Histogram showing the number of colonies formed by unsorted (dark grey column) and early adherent H357 cells (light grey column) after 14 days. All the assays were performed in triplicate and repeated three times. The error bar represents SEM. (p-value<0.001).

4.4.5 Determination of extracellular vesicles derived from early adherent and chemoresistant cells

In current experiment, the number of EVs derived from isolated cells by the two methods (rapid adhesion to fibronectin and chemoresistance to cisplatin) were investigated and compared with that derived from unsorted cells.

Analysis of our results showed that the number of EVs in the conditioned media collected from early adherent cells for both examined cell lines (H357 and SCC4) were significantly higher than that derived from unsorted, figures 4.15 and 4.16.

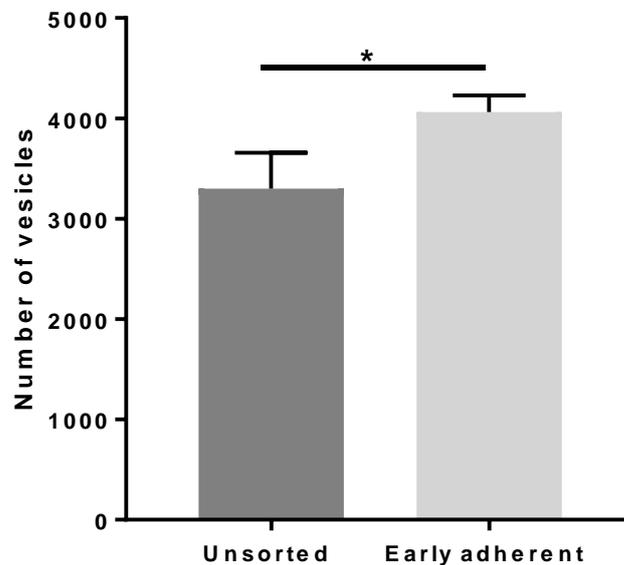


Figure 4.15: The number of extracellular vesicles EVs (50-150nm in diameter) were counted using an IZON Qnano machine of the conditioned media collected from unsorted (dark grey column) and early adherent H357 cells (light grey column). All the assays were performed in triplicate and repeated three times. The error bar represents SEM. ($P \leq 0.05$).

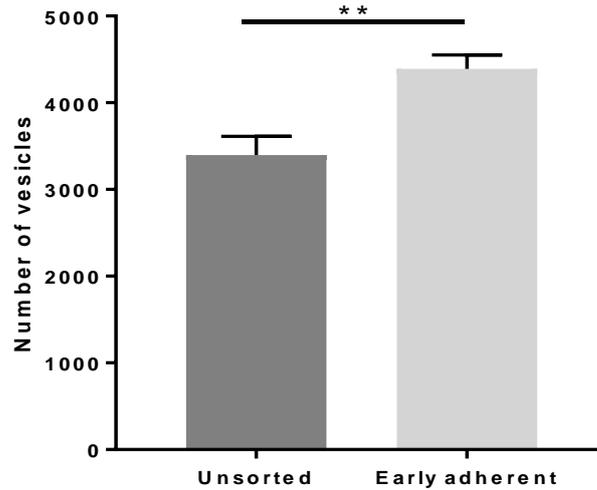


Figure 4.16: The number of extracellular vesicles EVs (50-150nm in diameter) were counted using an IZON Qnano machine of the conditioned media collected from unsorted (dark grey column) and early adherent SCC4 cells (light grey column). All the assays were performed in triplicate and repeated three times. The error bar represents SEM. ($P \leq 0.05$).

Chemoresistant cells isolated from H357 and SCC4 cancer cell lines exhibited a much larger increase in the levels of secreted extracellular vesicles in comparison to that of unsorted cells, figure 4.17 and 4.18.

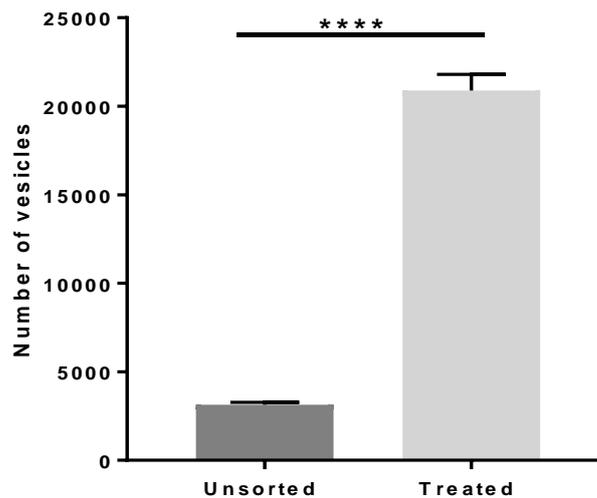


Figure 4.17: The number of extracellular vesicles EVs (50-150nm in diameter) counted using an IZON Qnano machine of the conditioned media collected from unsorted (dark grey column) and chemoresistant H357 cells (light grey column). All the assays were performed in triplicate and repeated three times. The error bar represents SEM. ($P \leq 0.0001$).

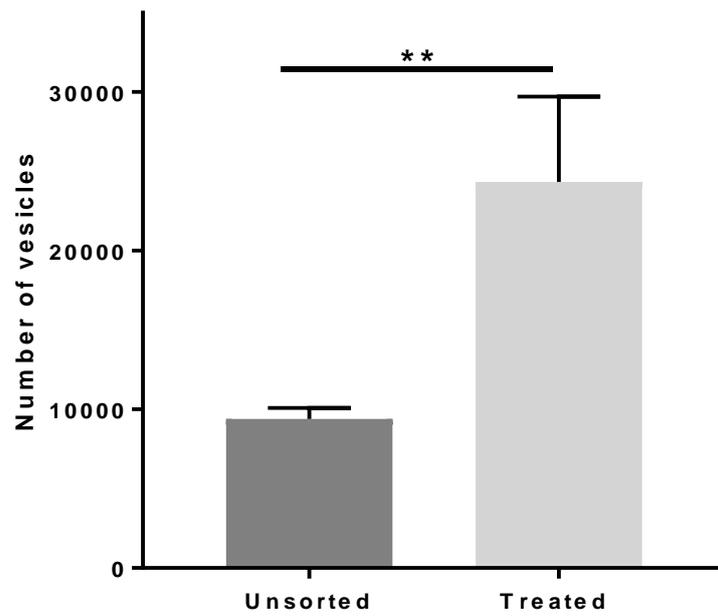


Figure 4.18: The number of extracellular vesicles EVs (50-150nm in diameter) counted using an IZON Qnano machine of the conditioned media collected from unsorted (dark grey column) and chemoresistant SCC4 cells (light grey column). All the assays were performed in triplicate and repeated three times. The error bar represents SEM. ($P \leq 0.001$).

4.5 Discussion

It has been established that a tumour consists of a heterogeneous population of cells with CSCs representing a minor subset of specialised cells with unique biological characteristics and distinct cell surface protein and gene expression that have a significant role in the enhancement of pro-malignant features. Characterisation approaches of CSCs generally are based on investigation of unique functional properties as well as particular surface markers that differentiate them from non CSCs (Dobbin and Landen, 2014). Although, many *in vitro* and *in vivo* assays have been developed to characterise CSCs, there is no method that can exclusively isolate or verify CSCs (Tirino *et al.*, 2012) and there are advantages and disadvantages of each experimental method (Islam, Gopalan *et al.*, 2015). It has been reported that FACS analysis represents an advanced, sensitive and specific technique of sorting and characterising of CSCs (Clarke *et al.*, 2006). However, it has some disadvantages such as; it is an expensive method and requires a high skilled and well trained user (Walia and Elble, 2010). Moreover, there are several technical limitations that affect the accuracy of FACS results. For instance the process of the preparing cell suspension (especially for solid tumours), setting of the gates and the quality of antibodies used in different procedures (Clarke *et al.*, 2006). However, it has been established that utilisation of a set of specific phenotypic and functional cell surface markers is crucial to obtain an unequivocal characterisation of CSCs (Tarnok, Ulrich and Bocsi, 2010). CSCs in many tumour types share several specific cell surface markers (Mannelli and Gallo, 2011).

On the other hand, many reports emphasise that the xenotransplantation assay is a gold standard assay for identifying and characterising of CSCs. This is because of it is a functional assay based on the tumorigenic characteristics of CSC and their ability to initiate a secondary tumour when transplanted into immunodeficient host with a phenotypic heterogeneity similar to that of original tumour (Tirino *et al.*, 2012; Dobbin and Landen, 2014). Although, this assay is considered a very sensitive *in vivo* assay for detection of CSCs (Biddle and Mackenzie, 2012), it is influenced by several factors that should be considered. For instance: number of injected cells i.e. tumour are formed at a higher frequency with fewer CSCs, the mouse strain and lack of immune response (immune system completely knocked-out) (Rosen and Jordan, 2010; Dobbin and Landen, 2014). In addition, the site of injection i.e. if the cells are

directed to a niche which might preferable than subcutaneous xenografts (O'Brien, Kreso and Jamieson, 2010). Several studies have been conducted on HNSCC cell lines that using an *in vivo* xenotransplantation assay to characterise oral CSCs (Prince *et al.*, 2007; Chen *et al.*, 2009; Zhang *et al.*, 2009; Liang *et al.*, 2014).

It is evident that most literature recommends that the most reliable method to characterise an isolated CSC population should examine a combination of surface cell markers and gene profile followed by investigation of functional characteristics (Dangles-marie *et al.*, 2007; Masahiko *et al.*, 2012; Ray *et al.*, 2012; Rao *et al.*, 2013). Therefore, in this chapter we firstly investigated the expression of the most common CSC surface markers in head and neck squamous cell carcinoma and their related genes as well as the most important *in vitro* functional assays of the isolated population of cells using both isolation methods.

FACS analysis was the first step in the characterisation of our sorted population of cells after isolation using early adhesion to fibronectin and chemoresistance to cisplatin. Selection of appropriate markers for characterisation of sorted population of cells in CSCs studies is mainly depended on the specific tumour type (Dobbin and Landen, 2014). Therefore, we selected a set of three of the most common head and neck squamous cell carcinoma cell surface markers CD44 (hyaluronate receptor), CD24 (specific ligand of P-selectin receptor) and CD29 (integrin- β 1 receptor). Our selection of multiple markers is due to the fact that there is no universal or single specific marker for CSCs and there is variation between tumour types as to the markers which best distinguish CSCs from non-CSCs populations (Tirino *et al.*, 2012). In addition, CSCs can not be discriminated using a single cell surface marker because the patterns of marker expression partly overlap between cell lineages (Tarnok, Ulrich and Bocsi, 2010). Furthermore, sorting and / or characterising of CSCs using a combination of stem cell surface markers was considered more reliable method yielding a more purified population of CSCs than using a single marker alone (Han *et al.*, 2014). Our findings of FACS analysis of sorted and unsorted cells of the both tested oral cancer cell lines (H357 and SCC4) isolated by the two different functional methods (adhesion and chemoresistance) showed that the isolated cells expressed significantly increased levels of all investigated stem cell surface markers (CD24, CD44 and CD29) compared to that of unsorted cells. This is consistent with

studies by Han *et al.*, (2014) which showed a population of CD24⁺/CD44⁺ head and neck squamous cell carcinoma cells expressed higher levels of CD29 and possessed 'stemness' characteristics. Other studies on colon cell lines reported that sorted CSCs populations expressed significantly higher levels of CD24, CD44 and CD29 (Vermeulen *et al.*, 2008; Kemper, Grandela and Medema, 2010). In addition, many studies reported at least two markers from the panel of surface markers used in our work (CD24, CD44 and CD29), were highly expressed by CSCs. For instance, some studies showed CSCs with increased levels of CD24 and CD44 (Li *et al.*, 2007; Han *et al.*, 2009; Dembinski and Krauss, 2009; Abubaker *et al.*, 2013). Moreover, Harper *et al.*, (2007) isolated CSCs from HNSCC cell lines and Geng *et al.*, (2013) from skin SCC using high expression levels of a combination of CD44 and CD29. Whereas, some studies identified that CSCs exhibited overexpression levels of both CD24 and CD29 (Shackleton *et al.*, 2006; Vassilopoulos *et al.*, 2008). The H357 cell line showed the biggest difference in the expression of CD44 stem cell marker between the isolated cells (early adherent and chemoresistant) and unsorted cells, while SCC4 cell line exhibited the biggest difference in expression of CD29 between isolated and unsorted cells in the two isolation methods (adhesion and chemoresistance). Variation in expression of stem cell markers between studies could be interpreted by individual differences among various cell lines or differences in the functional properties or composition of the CSC population (Barr *et al.*, 2013).

In the second experiment of our characterisation, qPCR was performed to investigate the expression of stem cell associated genes (CD24, CD44 and CD29), the cell surface protein expression of which was examined in the first set of experiments. The results of qPCR analysis demonstrated a significant up-regulation in the expression levels of all investigated genes (CD24, CD44 and CD29) in the both isolated populations of cells (early adherent and chemoresistant) of the both tested OSCC cell lines (H357 and SCC4). These results are consistent with the findings in the first experiment and suggest that these cells have the same pattern of surface protein and gene expression. This strong correlation that was found between expression of stemness associated genes and cell surface proteins of the isolated cell populations in this study confirms the 'stemness' features of the sorted early adherent and chemoresistant cells. Although previous literature did not investigate expression of CD24, CD44 and CD29 genes together in the characterisation of CSCs, some studies reported an up-regulation

of combination of CD24 and CD44 with other stemness associated genes such as: Han *et al.*, (2009) in their characterising of CSCs in HNSCC cell lines and this in agreement with our findings. However, there are many CSC characterising studies which identified increased levels of various stemness associated genes in CSC subpopulations and they reported up-regulation of at least one of the genes investigated in our study. For instance, Zhang *et al.*, (2009), Yeung *et al.*, (2010) and Abubaker *et al.*, (2013) demonstrated high levels of CD44 expression was associated other stem cell genes in sorted CSCs. Whereas, Gao *et al.* (2010) found an up-regulation level of CD24 in liver CSCs. In addition, Li *et al.*, (2005) and Liang *et al.*, (2014) reported increased levels of CD29 in CSCs .

Proliferation assays were performed as the third set of experiments in our characterisation of sorted cells. It was considered as one of important *in vitro* functional assays for characterising of CSCs, based on the CSCs hallmark of being as relatively quiescent cells with low mitotic activity (Roesch *et al.*, 2010). The low growth rate of CSCs was considered an important conventional anticancer therapy mechanism that plays a crucial role in avoiding the effects of treatment (Maugeri-Saccà, Zeuner and De Maria, 2011). The results of proliferation assays in current study indicated a significant reduced growth rate of sorted cell subpopulations (early adherent and chemoresistant) compared to unsorted cells of both examined OSCC cell lines (H357 and SCC4). These findings in accordance with that established in previous studies (Dallas *et al.* 2009; Gao *et al.*, 2010; Barr *et al.*, 2013; Han *et al.*, 2014).

The other functional assay that was conducted in this study to characterise the cell populations after isolation by early adhesion and chemoresistance was a colony forming assay. This assay is based on the enhanced ability of CSCs to form a colony from single cell, which is considered one of unique intrinsic properties of CSCs (Rodermond *et al.*, 2006). In general, the size and number of the colonies originate from CSCs are greater than those formed from non-CSCs (Tirino *et al.*, 2012). Furthermore, Locke *et al.*, (2005) and Harper *et al.*, (2007) indicated in their studies that clonogenicity was limited to a CSC population within the total population of cells from HNSCC cell lines and that these were also associated with specific cellular morphology and distinct surface markers expression as well as cellular heterogeneity which reflected the pattern of stem cell hierarchy within HNSCC cell lines. Our

evidence from colony forming assays revealed that early adherent cells of both tested OSCC cell lines showed a significantly higher clonogenic potential through formation of more colonies compared to that formed by the same number of unsorted cells. This is in agreement with previous studies on HNSCC cell lines (Locke *et al.*, 2005; Harper *et al.*, 2007; Zhang *et al.*, 2009; Han *et al.*, 2014; Liang *et al.* 2014). In addition, studies of other tissues show that this is also the case for skin squamous cell carcinoma (Geng *et al.*, 2013), lung CSCs (Barr *et al.*, 2013), pancreatic CSCs (Collins *et al.*, 2005), colon CSCs (Dalas *et al.*, 2009) and breast CSCs (Vassilopoulos *et al.*, 2008). On the other hand, the failure of formation of colonies by chemoresistant cells after incubation for 14 days might be attributed to the delayed action of cisplatin and the fact that such cells carry on dying sometime after the drug has been removed.

The last step of experiments was conducted to quantify the number of EVs derived from both isolated cells (early adherent and chemoresistant) and unsorted cells. It is evident that crosstalk between heterogeneous population of cells within a tumour are mediated by soluble molecules like: growth factors, cytokines, chemokines and proteinases (Hanahan and Coussens, 2012). However, recent studies report that extracellular vesicles (EVs) could be a major mechanism of mediation of cellular interactions in the tumour microenvironment (Roma-rodrigues, Fernandes and Baptista, 2014).

Our findings revealed that chemoresistant cells of both examined cell lines (H357 and SCC4) secreted significantly enhanced number of EVs compared to that released by unsorted cells. This result is in agreement with that reported by Samuel *et al.*, (2018) in their study on ovarian cancer cell where it was established that treatment with 20 μ M cisplatin for 3 hours and after 3 days recovery time was enhanced secretion of cancer cells derived EVs. Whilst early adherent cells here shown to be CSC like, there are no previous reports of early adherent cells secreting enhanced levels of EVs.

4.6 Conclusions

The consistency of results of all characterisation experiments that were performed on early adherent and chemoresistant cells of the both tested oral cancer cell lines (H357 and SCC4) which showed expression of enhanced levels of stem associated genes

with their related stem cell surface marker proteins, low proliferation rate, high clonogenic potential and secretion of EVs confirms the ‘stem-like’ characteristics of both isolated populations of cells. In addition, our findings in previous chapter of the strong correlation between the isolated populations suggest the two methods isolate phenotypically overlapping populations of CSCs.

CHAPTER 5

ROLE OF CSCs IN

ACTIVATION OF STROMAL

FIBROBLASTS

5.1 Introduction

The tumour microenvironment of solid tumours consists mainly of two fundamental elements which are the neoplastic epithelial cells and several types of host stromal cells that have great influence on the growth and invasion of cancer cells (Liotta and Kohn, 2001; Kalluri and Zeisberg, 2006; Joyce and Pollard, 2009) .

Although, fibroblasts are considered the most abundant cells within a stroma that provide niches and mechanical support for cancer cells. Cancer associated fibroblasts (CAFs) represent an activated fibroblast phenotype and the most common and important one in the tumour playing pivotal roles in tumourgenesis. These roles include: stimulation of tumour cell proliferation, modulation of immune responses, resistance to therapies as well as promotion of angiogenesis, metastasis and invasion (Ishii *et al.*, 2016). This may occur as a result of their secretion of ECM proteins and their proteases as well as soluble factors such as inflammatory cytokines and growth factors (Ilzle *et al.*, 2004; Kalluri and Zeisberg, 2006).

It has been reported that normal fibroblasts in the tumour stroma acquire a CAF phenotype and are activated due to signals released from cancer cells as a part of what is known stromal-tumour crosstalk (Elenbaas and Weinberg, 2001; Wever and Mareel, 2003; Kalluri and Zeisberg, 2006).

TGF- β 1 is a well-studied potent activating factor of normal fibroblasts transforming them into a myofibroblast phenotype with enhanced expression of α -SMA (Honda, Yoshida and Munakata, 2010). In addition, previous work in our lab established that normal fibroblasts treated with 10ng/mL TGF- β 1 for 48 hours is a good model of activating fibroblasts and as a consequence we used this as positive control.

Therefore, in this chapter we examined the influence of the conditioned media derived from the early adherent and chemoresistant cells on the activation of normal oral fibroblasts (NOFs). This was performed through investigating CAF characteristics features and gene profile in NOFs activated by isolated and unsorted cells and comparing them with the findings of untreated NOFs and those activated with TGF- β 1.

5.2 The aims of the chapter

1. To investigate the expression of α - smooth muscle actin protein fibres (α -SMA) in NOFs incubated with conditioned media from isolated (early adherent and chemoresistant) and unsorted cells using immunofluorescence. As a result of abundant expression of α -SMA by activated fibroblasts this is considered a crucial hallmark discriminating them from quiescent fibroblast (Pourreyron *et al.*, 2003; Kalluri and Zeisberg, 2006; Kellermann *et al.*, 2008).
2. To investigate the expression of some CAF associated genes (α -SMA and IL-6) in the NOFs incubated with conditioned media of isolated and unsorted cells.

5.3 Methods

The following methods were used in this chapter:

- Cell culture (section 2.2)
- Isolation of early adherent cells by adhesion assay (section 2.3)
- Isolation of chemoresistant cells by chemoresistance assay (section 2.5)
- Activation of NOFs by early adherent and chemoresistant cells (section 2.17)
- Immunofluorescence (section 2.16)
- RNA extraction from isolated and unsorted cells (section 2.12)
- cDNA synthesis (section 2.13)
- Real time –Polymerase chain reaction (qPCR) (section 2.14)

5.4 Results

5.4.1 Effects of conditioned media from isolated cells on normal oral fibroblast expression of α - smooth muscle actin fibres.

In this experiment, immunofluorescence staining using anti- alpha smooth muscle actin antibody was used to investigate the expression of α - smooth muscle actin (α -SMA) protein fibres in normal oral fibroblasts (NOFs) after their incubation for 48 hours with condition media derived from sorted (early adherent or chemoresistant) and unsorted cells.

Our results showed that the NOFs that were incubated in conditioned media collected from early adherent cells of both tested oral cancer cell lines (H357 and SCC4) stimulated and exhibited significantly more intense α -SMA staining compared to the NOFs incubated with conditioned media derived from unsorted cells, figures 5.1 and 5.2.

Similarly, significant more intense α -SMA staining was observed in NOFs when they were grown in conditioned media derived from chemoresistant H357 and SCC4 oral cancer cell lines in comparison to that expressed by NOFs incubated with conditioned media of unsorted cells, figures 5.3 and 5.4.

In addition, in all our immunofluorescence experiments the NOFs exposed to media alone showed very low α -SMA staining, while NOFs treated with 10ng/mL TGF- β 1 were exhibited very obvious α -SMA staining, figures 5.1, 5.2, 5.3 and 5.4.

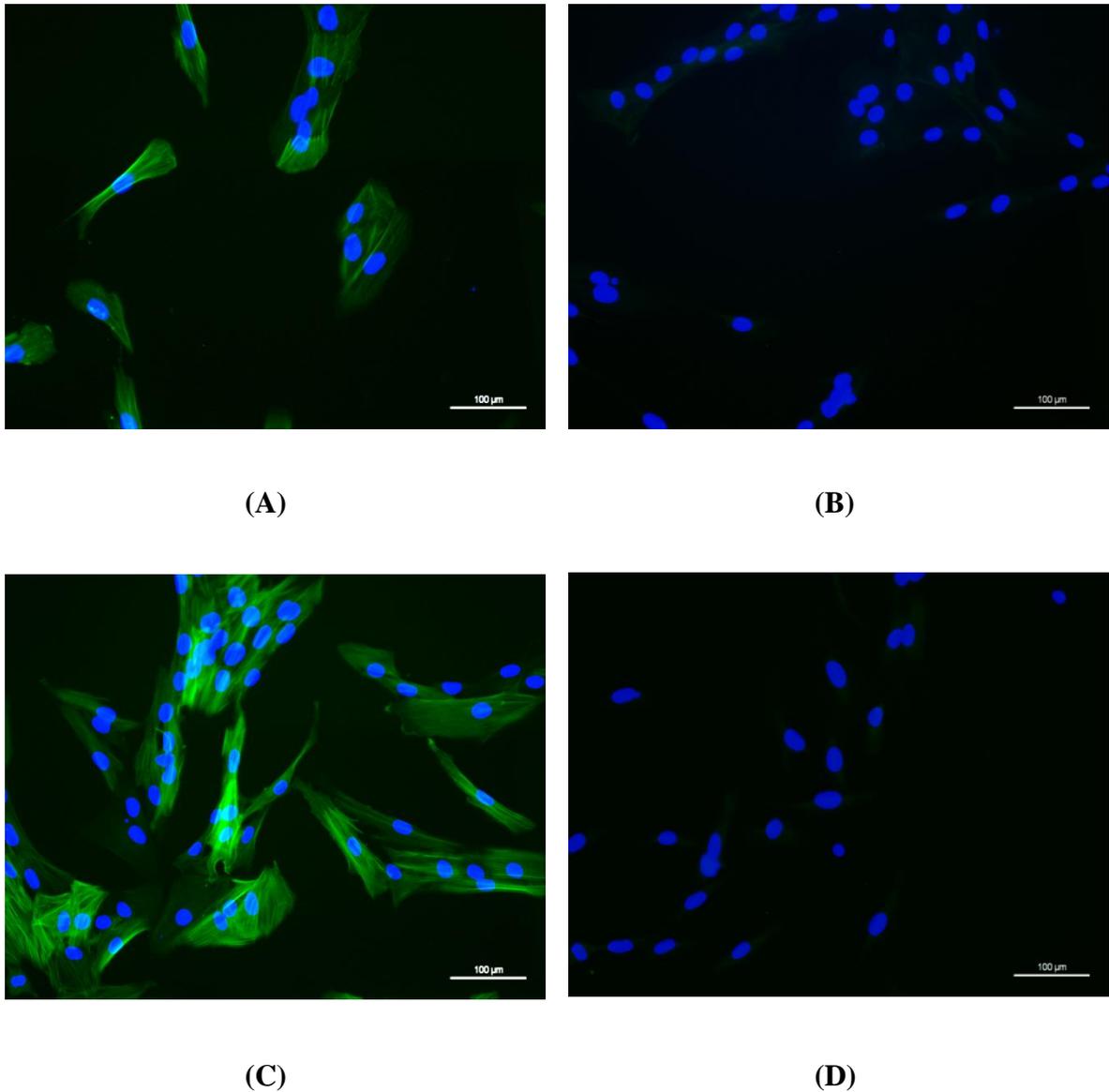


Figure 5.1: Representative photomicrographs of α -SMA immunofluorescence in normal oral fibroblasts (NOFs). NOFs were treated for 48 hours and the treatment consisted of (A) conditioned media derived from early adherent H357 cells (B) conditioned media derived from unsorted cells of H357 cell line (C) with 10 ng/mL of TGF- β 1 (D) without treatment. The cell nucleus is stained in blue (DAPI) and the α -SMA fibres in green (FITC). (Magnification: 200 \times).

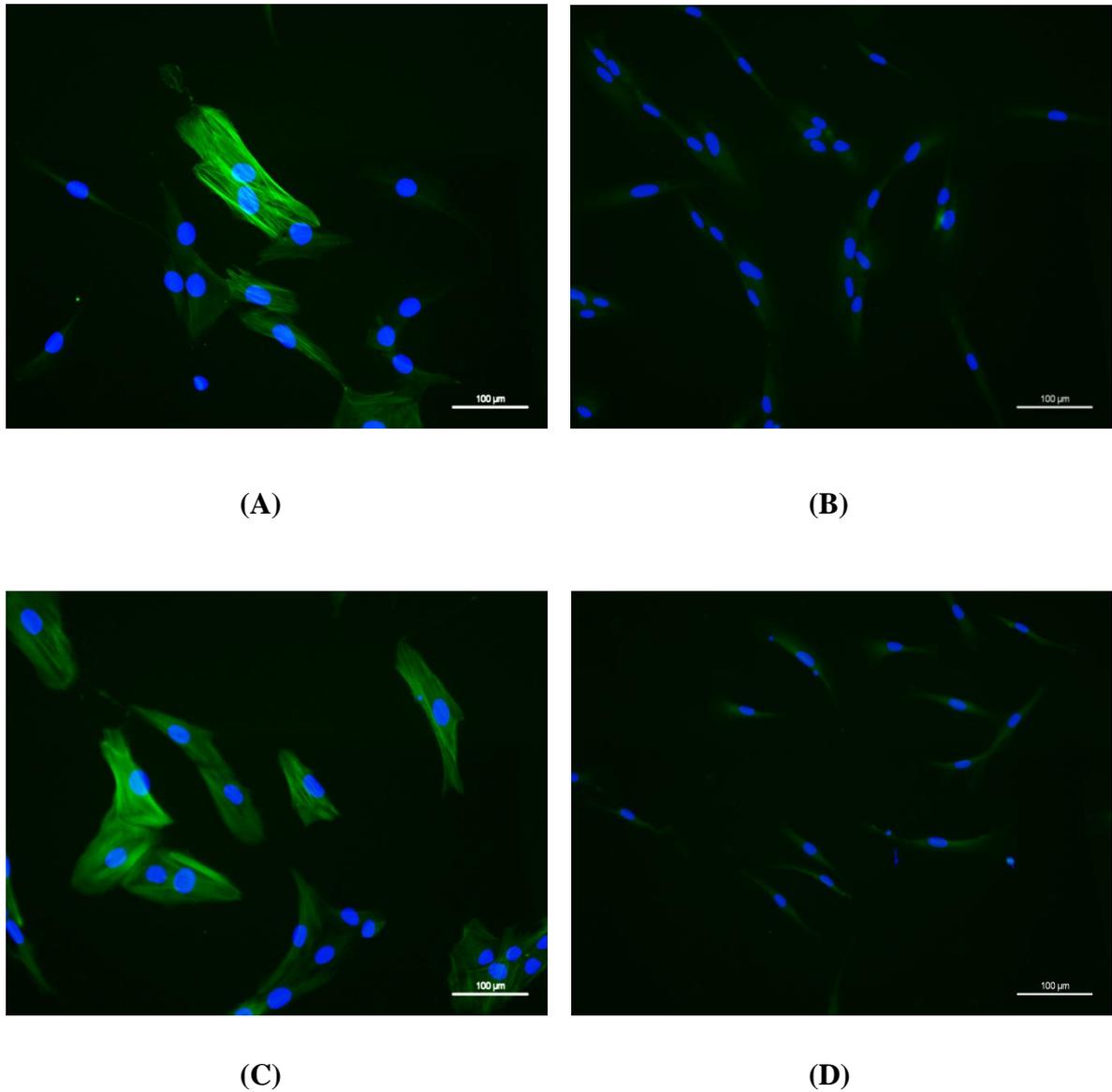


Figure 5.2: Representative photomicrographs of α -SMA immunofluorescence in normal oral fibroblasts (NOFs). NOFs were treated for 48 hours and the treatment consisted of (A) conditioned media derived from early adherent SCC4 cells (B) conditioned media derived from unsorted cells of SCC4 cell line (C) with 10 ng/mL of TGF- β 1 (D) without treatment. The cell nucleus is stained in blue (DAPI) and the α -SMA fibres in green (FITC). (Magnification: 200 \times).

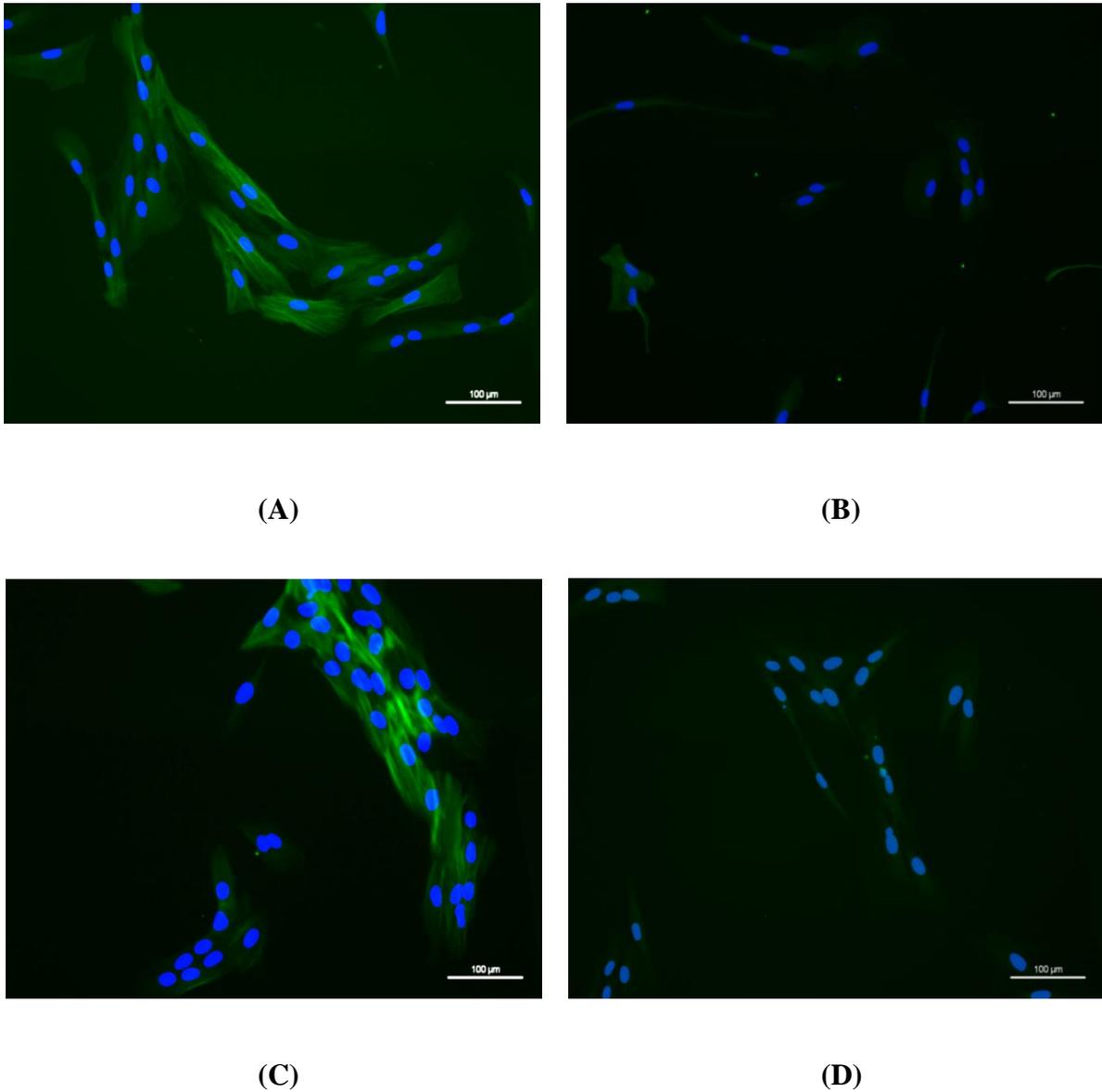


Figure 5.3: Representative photomicrographs of α -SMA immunofluorescence in normal oral fibroblasts (NOFs). NOFs were treated for 48 hours and the treatment consisted of (A) conditioned media derived from chemoresistant H357 cells (B) conditioned media derived from unsorted cells of H357 cell line (C) with 10 ng/mL of TGF- β 1 (D) without treatment. The cell nucleus is stained in blue (DAPI) and the α -SMA fibres in green (FITC). (Magnification: 200 \times).

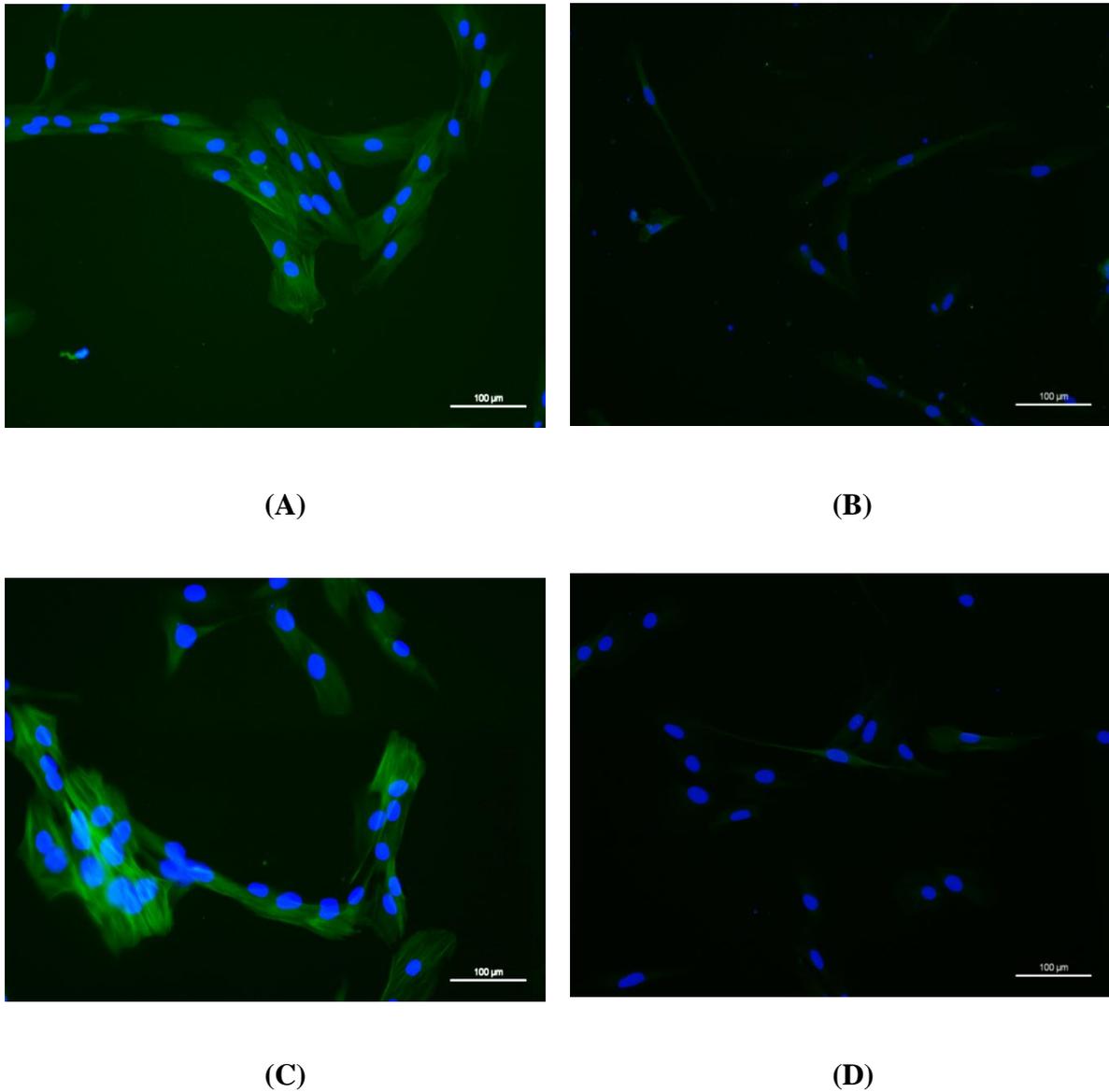


Figure 5.4: Representative photomicrographs of α -SMA immunofluorescence in normal oral fibroblasts (NOFs). NOFs were treated for 48 hours and the treatment consisted of (A) conditioned media derived from chemoresistant SCC4 cells (B) conditioned media derived from unsorted cells of SCC4 cell line (C) with 10 ng/mL of TGF- β 1 (D) without treatment. The cell nucleus is stained in blue (DAPI) and the α -SMA fibres in green (FITC). (Magnification: 200 \times).

5.4.2 Assessment of α - smooth muscle actin gene expression in normal oral fibroblasts.

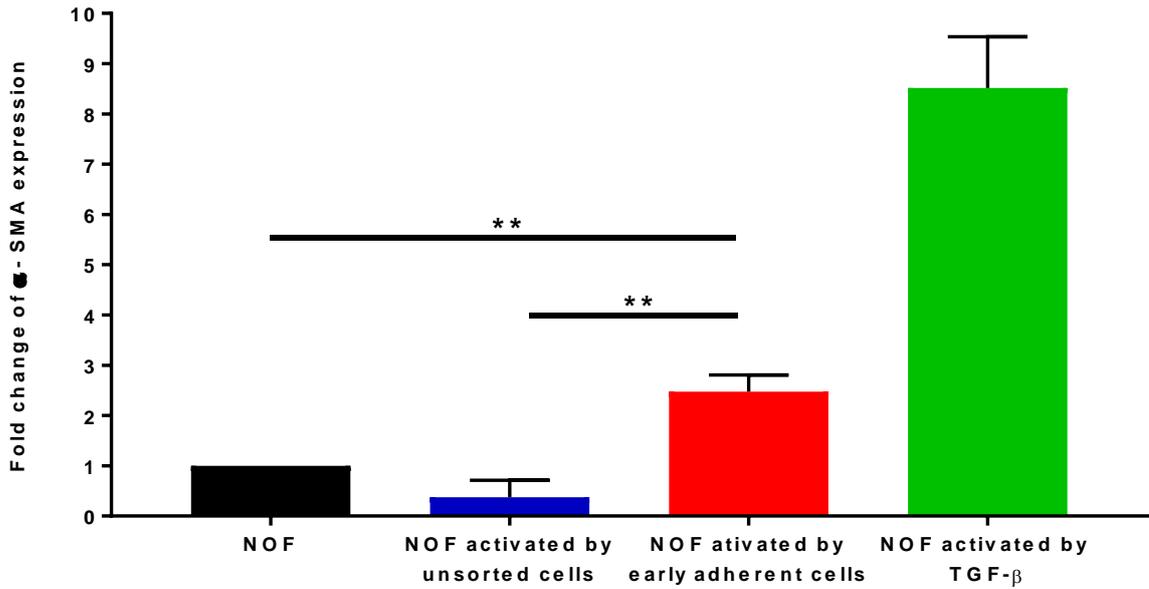
In order to quantify the increase in α -SMA observed by immunofluorescence experiments, qPCR analysis were performed to investigate the level of α - smooth muscle actin gene (α -SMA) expression in normal oral fibroblasts exposed to conditioned media derived from cells sorted by adhesion and chemoresistance from both oral cancer cell lines and compared to that of unsorted cells.

The levels of α -SMA expressed by NOFs treated with 10 ng/mL TGF- β 1 was used as positive control, while that of those treated with media alone as negative control.

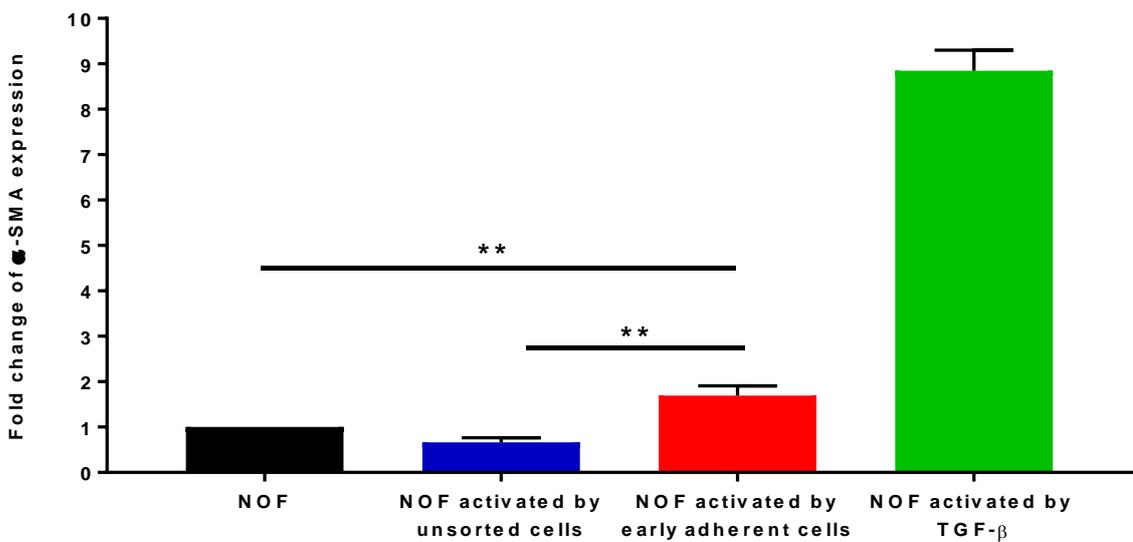
Our results indicated that NOFs stimulated with conditioned media derived from both isolated cells (early adherent and chemoresistant cells) of both tested oral cancer cell lines (H357 and SCC4) expressed significantly up-regulated levels of α -SMA which were 2-3 fold higher compared to that expressed by untreated NOFs and NOFs stimulated by conditioned media from unsorted cells, figures 5.5 and 5.6.

On the other hand, NOFs treated with conditioned media derived from unsorted cells of both OSCC cell lines (H357 and SCC4) exhibited less levels of α -SMA than that of untreated NOFs, figures 5.5 and 5.6.

In addition, NOFs treated with 10 ng/mL TGF- β 1 showed very obviously raised levels of α -SMA gene expression (8-9 fold) compared to that from NOFs stimulated by conditioned media from sorted or unsorted cells as well as untreated NOFs in all experiments, figures 5.5 and 5.6.

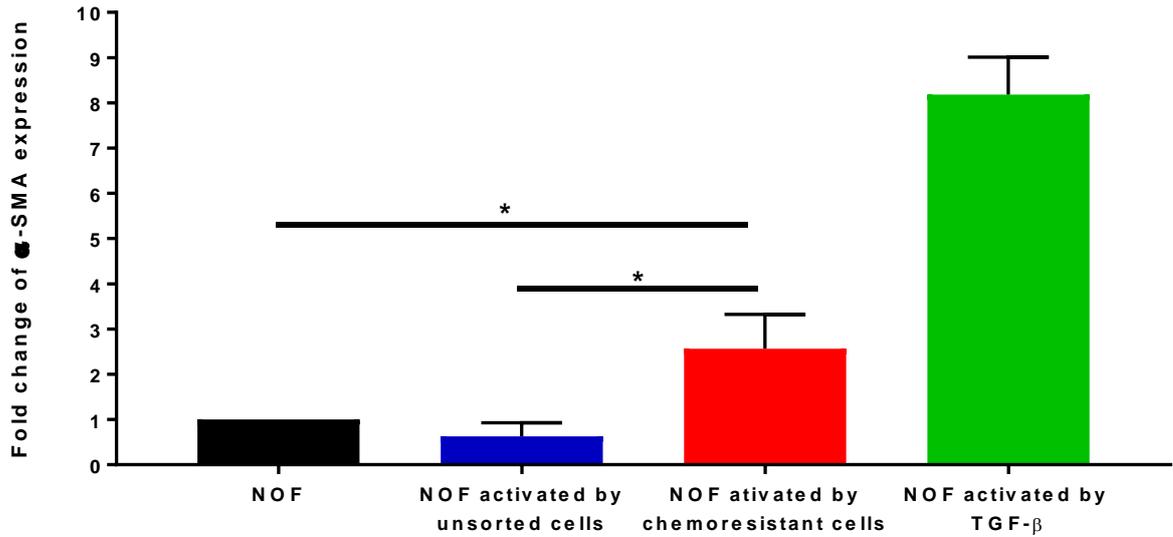


(A) H357 cell line

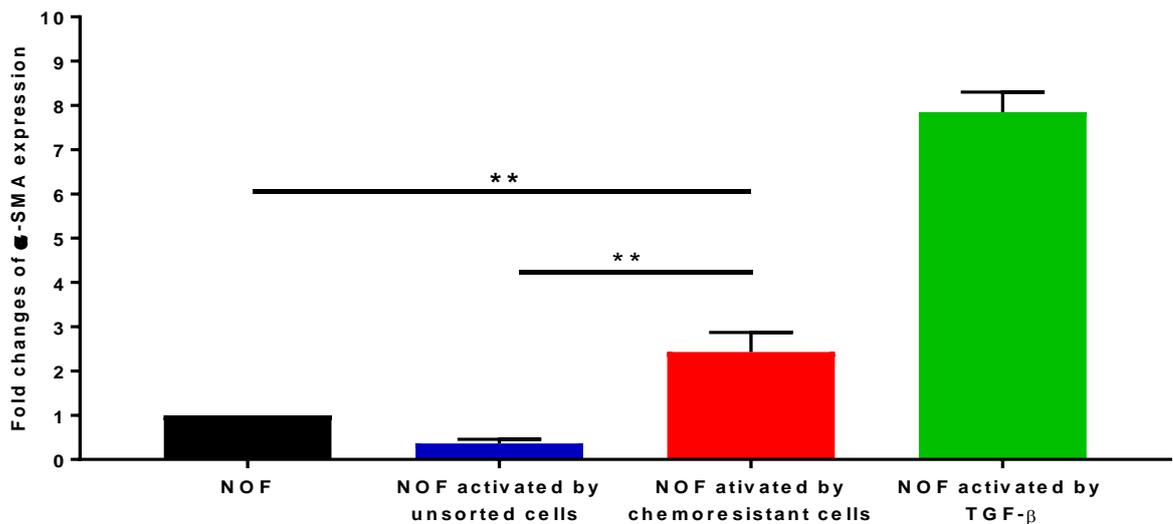


(B) SCC4 cell line

Figure 5.5: The mean fold change in α -SMA gene expression by normal oral fibroblasts (NOFs) treated with conditioned media derived from early adherent cells (red bars) relative to the non-treated NOFs control (black bars) (A) H357 cell line (B) SCC4 cell line. NOFs treated with conditioned media derived from unsorted cells (blue bars), NOFs treated with 10 ng/mL of TGF- β 1 (green bars). \pm SD of at least 3 independent experiments performed in triplicate. *, ** Indicate statistically significant differences at $P \leq 0.05$ and $P \leq 0.001$ respectively.



(A) H357



(B) SCC4

Figure 5.6: The mean fold change in α -SMA gene expression by normal oral fibroblasts (NOFs) treated with conditioned media derived from chemoresistant cells (red bars) relative to the non-treated NOFs control (black bars) (A) H357 cell line (B) SCC4 cell line. NOFs treated with conditioned media derived from unsorted cells (blue bars), NOFs treated with 10 ng/mL of TGF- β 1 (green bars). \pm SD of at least 3 independent experiments performed in triplicate. *, **, ** Indicate statistically significant differences at $P \leq 0.05$, $P \leq 0.001$ and $P \leq 0.0001$ respectively.**

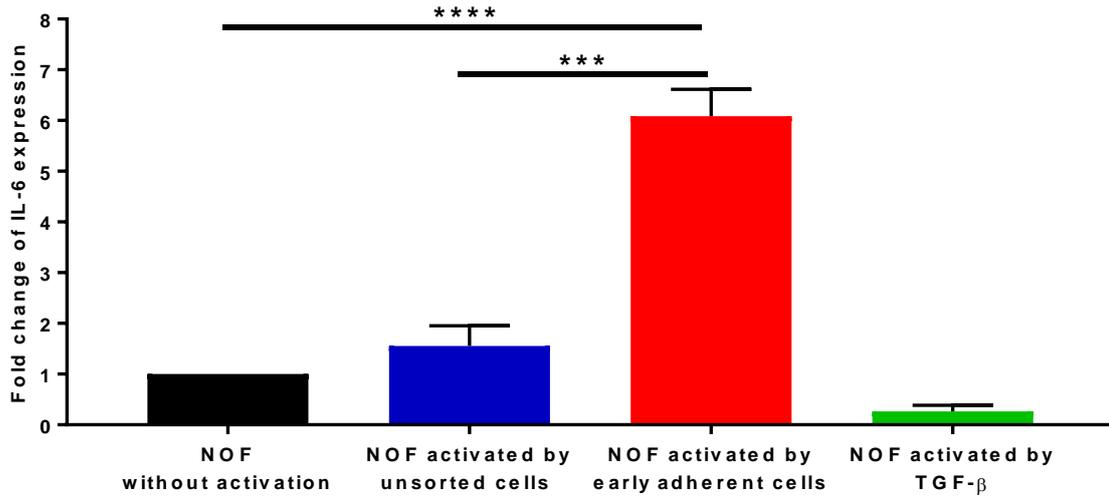
5.4.3 Assessment of interleukin-6 (IL-6) gene expression in stimulated normal oral fibroblasts.

Further assessment of the normal oral fibroblast gene expression profile using qPCR was performed in cells stimulated with conditioned media derived from early adherent and chemoresistant cells to investigate the expression of IL-6 which is considered one of common up-regulated proinflammatory genes in CAFs (Erez et al., 2010). Expression was compared with that of untreated NOFs as well as with NOFs incubated with conditioned media of unsorted cells.

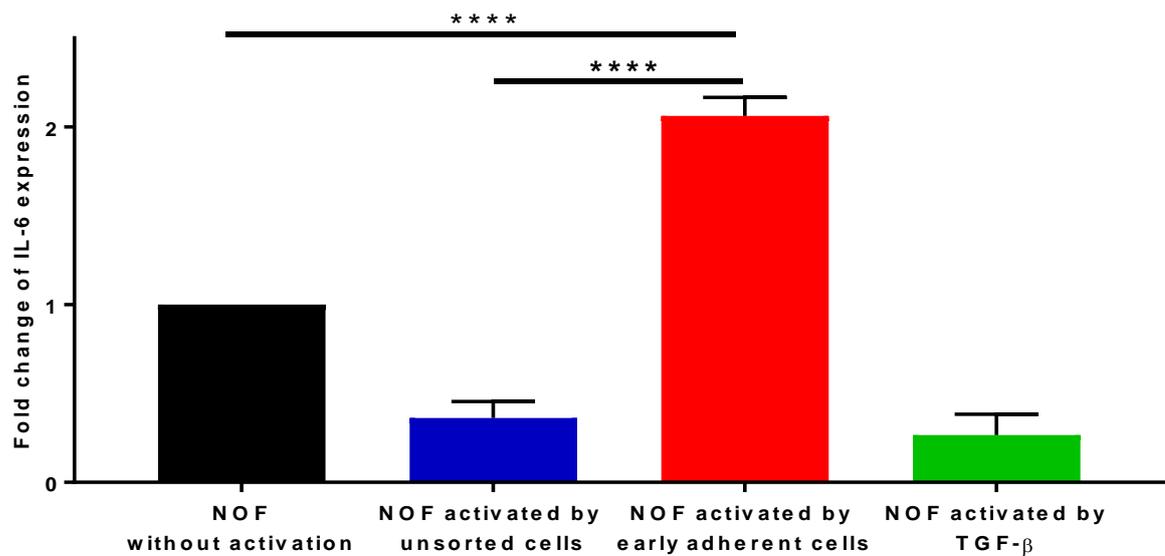
Our findings revealed a significant up-regulation of IL-6 expression levels in NOFs exposed to conditioned media collected from isolated cells from both OSCC cell lines (H357 and SCC4) whether they were sorted by rapid adhesion or by chemoresistance. The fold changes were nearly 5 fold in the expressions of IL-6 by NOFs triggered by conditioned media derived from early adherent and chemoresistant cells of H357 cell line in comparison to the levels of IL-6 expressed by NOFs grown in conditioned media of unsorted cells and untreated NOFs. Whereas, it was 2 fold higher in NOFs incubated in conditioned media of early adherent and chemoresistant cells of SCC4 cell lines compared to the levels of IL-6 expressed by NOFs grown in conditioned media of unsorted cells and untreated NOFs, figures 5.7 and 5.8.

Furthermore, IL-6 levels expression of NOFs incubated in conditioned media from unsorted SCC4 cells showed down-regulation in levels compared to that expressed by non-treated NOFs, figures (5.7 B) and (5.8 B).

NOFs treated with 10 ng/ml TGF- β 1 showed very obviously down-regulated levels of IL-6 gene expression compared to that from NOFs stimulated by conditioned media from sorted or unsorted cells as well as untreated NOFs in all experiments, figures 5.7 and 5.8.

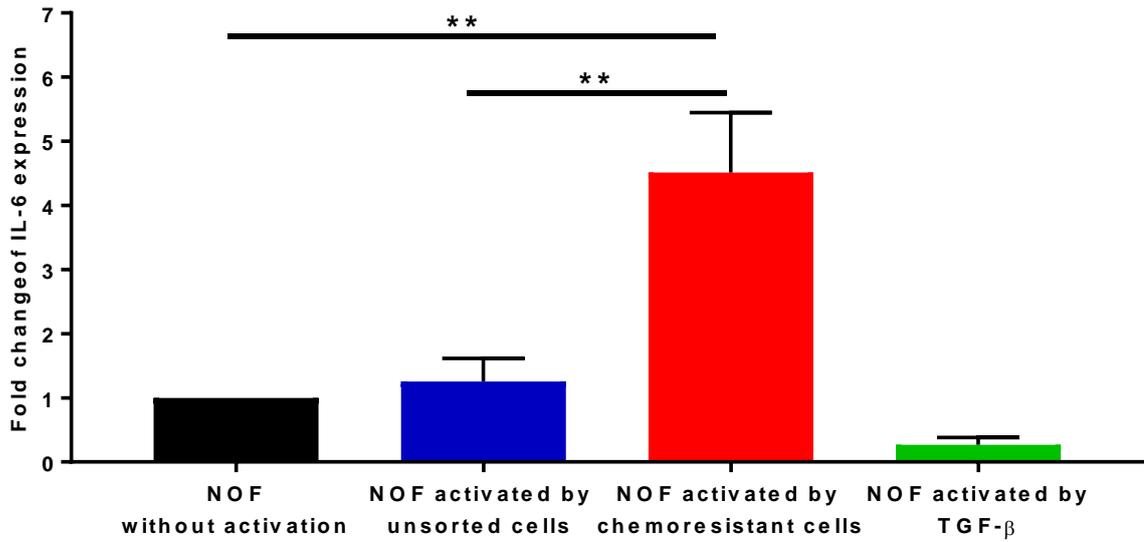


(A) H357 cell line

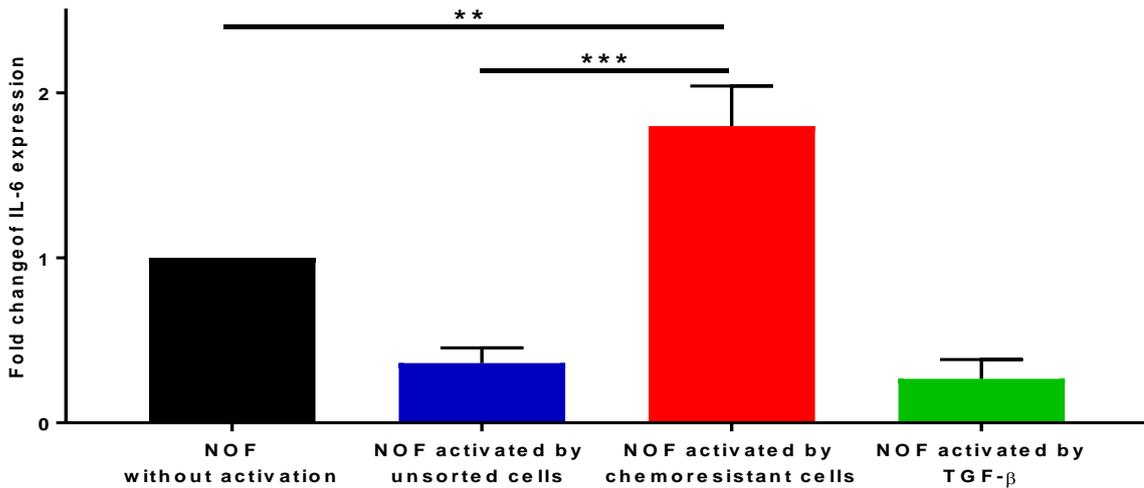


(B) SCC4 cell line

Figure 5.7: The mean fold change in IL-6 gene expression in normal oral fibroblasts (NOFs) treated with conditioned media derived from early adherent cells (red bars) relative to the non-treated NOFs control (black bars) (A) H357 cell line (B) SCC4 cell line. NOFs treated with conditioned media derived from unsorted cells (blue bars), NOFs treated with 10 ng/mL of TGF- β 1 (green bars). \pm SD of at least 3 independent experiments performed in triplicate. *, **** Indicate statistically significant differences at $P \leq 0.0001$.**



(A) H357 cell line



(B) SCC4 cell line

Figure 5.8: The mean fold change in IL-6 gene expression in normal oral fibroblasts (NOFs) treated with conditioned media derived from chemoresistant cells (red bars) relative to the non-treated NOFs control (black bars) (A) H357 cell line (B) SCC4 cell line. NOFs treated with conditioned media derived from unsorted cells (blue bars), NOFs treated with 10 ng/mL of TGF- β 1 (green bars). \pm SD of at least 3 independent experiments performed in triplicate. **, *** Indicate statistically significant differences at $P \leq 0.001$ and $P \leq 0.0001$ respectively.

5.5 Discussion

In the 1880s, Paget proposed the ‘seed and soil theory’ which considered that the tumour microenvironment was basically composed from seeds ‘the cancer cells’ and soil ‘stromal cells and ECM’ (Paget, 1889). Currently, it has been understood that tumour cells were modified genetically or epigenetically due to thorough molecular analysis of cancer cells which resulted in identification and characterisation of many oncogenes and tumour suppressor genes. On the contrary, there is no full characterisation for ‘soil’ of tumour microenvironment that is formed by both tumour and host stromal cells, probably due to its functional and structural complexity. Recently, there is increasing evidence that host stromal cells characteristics have great influence on the biological behaviour of tumour cells (Bhowmick *et al.*, 2004).

The tumour microenvironment consists of extracellular matrix (ECM) including mainly collagen, fibronectin, tenascin and laminin as well as different types of cells such as mesenchymal stem cells (George, Singh and Nirmala, 2012) and vasculature which includes endothelial cells, pericytes and smooth muscle cells (Polyak, Haviv and Campbell, 2009). In addition, inflammatory and immune cells are present recruited by cytokines and chemokines (Müller-hübenthal *et al.*, 2009) as well as various types of fibroblasts (Lorusso and Rüegg, 2008).

Head and neck squamous cell carcinoma is derived from epithelial cells, which are normally separated from the underlying connective tissue by a basal lamina (Weinberg, 2007). During tumour development, basement membrane is degraded and cancer cells invade into the connective tissue and become directly in contact with the stromal cells (Akashi *et al.*, 2005).

It has been reported that the growth of a tumour relies mainly on its genetic exclusivity and the interaction between cancer cells with stromal cells in tumour microenvironment (Galiè *et al.*, 2005; Kellermann *et al.*, 2008). In addition, stromal cells acquire a specific biological phenotype through their interaction with tumour cells (Bhowmick *et al.*, 2004). In turn, the activated stromal cells induce tumour cells through release of biological agents to promote growth and acquisition of an invasive phenotype as well as breakdown of the normal barrier created by the ECM (Hsu, Meier and Herlyn 2002; Wever and Mareel, 2003). Therefore, the crosstalk between

tumour cells and stromal cells plays a dynamic role in tumour initiation, survival, progression, invasion and resistance to conventional anticancer therapies (Patten *et al.*, 2008; Buggins *et al.*, 2010; Pepper *et al.*, 2011). This crosstalk is concentrated in two main interactive pathways: firstly, an efferent pathway by which cancer cells activate and/or modify the tumour leading to a reactive response. Whereas, the second pathway is an afferent one whereby modified and/or activated stromal fibroblasts cells in the tumour microenvironment affects the behaviour and responses of cancer cells (Wever and Mareel, 2003), as illustrated in figure 5.9.

Many soluble mediators have been reported to be involved in intercellular communication between tumour and stromal cells, such as: vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), transforming growth factor- β (TGF- β), hepatocyte growth factors (HGF), platelet derived growth factor (PDGF), insulin-growth factor-1 (IGF-1), stromal derived factor-1 (SDF-1) and ligands of the Wnt, Notch and Hedgehog pathways. In addition, these factors have important roles in tumour progression through induction of CSC self-renewal, tumour growth, angiogenesis, recruitment of stromal cells, invasion and metastasis (Molloy *et al.*, 2009; Qian *et al.*, 2012). Recently, reports established that cell derived exosomes are potent mediators of direct communication between cancer and stromal cells and that this can influence cancer cell survival, proliferation, invasion and metastasis (Camp *et al.*, 2011; Luga *et al.*, 2012).

Although evidence shows that fibroblasts form the most common stromal cell within tumour microenvironment, cancer associated fibroblasts (the activated phenotype of fibroblasts) are considered a key player in tumourigenesis (Cirri and Chiarugi, 2011; Öhlund, Elyada and Tuveson, 2014). One of the important hallmarks that discriminates CAFs from quiescent fibroblasts is the common expression of smooth muscle actin fibres (α -SMA) (Pourreyron *et al.*, 2003; Kalluri and Zeisberg, 2006; Kellermann *et al.*, 2008). It has been widely accepted that CAFs are histologically defined as plump, spindle shaped cells which positively expressed α -SMA, and are usually located at the tumour periphery (Thode *et al.*, 2011). Many studies have established that expression of α -SMA fibres as a myofibroblast ultra-structure within activated tumour stroma is considered the most prominent feature indicating phenotypic switching of quiescent fibroblasts into CAFs (Tuxhorn, Ayala and Rowley, 2001; Kunz-Schughart and Knuechel, 2002).

Our first finding that might suggest the activation of normal oral fibroblasts (NOFs) into CAFs when they were exposed to conditioned media from isolated cells (early adherent and chemoresistant) from the both tested OSCC cell lines (H357 and SCC4) was obtained from experiments assessing the immunofluorescent expression of smooth muscle actin fibres (α -SMA). The results showed highly intense staining of α -SMA in fibroblasts stimulated by media from isolated cells compared to no noticeable change in expression in those exposed to conditioned media from unsorted cells. This is in accordance with the findings of many studies which reported that CAFs were identified through their expression of α -SMA (Sugimoto *et al.*, 2006; Erez *et al.*, 2010; Wang *et al.*, 2013). Although, the very obvious difference in expression of α -SMA stain in NOFs activated by isolated and unsorted cells was observed by immunofluorescent microscopy, we could quantify this in future experiments using ImageJ software or extract protein from NOFs and perform western blotting for α -SMA.

CAFs promote and enhance cancer progression in different ways through secretion of various factors. CAFs mediate ECM remodelling through their abundant secretion and deposition of variety of ECM proteins such as collagen type I and IV, fibronectin, and tenascin-C leading to increased tumour growth. In turn, their secretion of proteases such as matrix metalloproteinases (MMPs) that digest ECM and have crucial roles in tumour progression, invasion and subsequent metastasis (Sato, Maehara and Goggins, 2004; Wever *et al.*, 2008; Kessenbrock, Plaks and Werb, 2010). CAFs secrete many pro-inflammatory cytokines and chemokines that recruit inflammatory cells and promote sustained inflammation in the tumour stroma as well as inducing neoangiogenesis through stimulation of pericytes and endothelial cells (Hanahan and Coussens, 2012). Moreover, they suppress recruitment of immune cells that results in modulation of the immune response (Misra *et al.*, 2008; Xiang *et al.*, 2009). Cancer cell proliferation is induced and regulated by growth factors such as hepatocyte growth factor (HGF) released from CAFs, as a result of the direct interaction and communication of cancer cells with CAFs (Tyan *et al.*, 2011; Jia *et al.*, 2013; Wu *et al.*, 2013; Saito *et al.*, 2015). Furthermore, EMT is induced in cancer cells by TGF- β secreted from CAFs (Yu *et al.*, 2014). In addition, the oncogenic potential of epithelial cells was modulated by TGF- β secreted from CAFs (Puisieux *et al.*, 2006). CAFs regulate CSC plasticity during tumour progression via a Nanog dependent

mechanism (Chen *et al.*, 2014). Although, the crucial role of CAFs in tumourigenesis and their obvious biological and clinical impacts in tumour microenvironments have been clearly recognised, the mechanism of activation of normal fibroblast to CAFs remains unclear (Lee *et al.*, 2015).

Previously, it was considered that CAFs just altered phenotypically but remained genetically similar to normal host derived cells. However, recent literature emphasised that CAFs were their genetic profile distinct from normal fibroblasts (Kurose *et al.*, 2001; Sugimoto *et al.*, 2006; Erez *et al.*, 2010). Moreover, it has been reported that α -SMA is a common marker for identifying CAFs (Tlsty, 2001; Tuxhorn *et al.*, 2001; Kunz-Schughart and Knuechel, 2002; Gabbiani 2003; Sugimoto *et al.*, 2006). In addition, there are other markers were overexpressed by CAFs such as IL-6 (Giannoni *et al.*, 2010; Osuala *et al.*, 2015; Yeh *et al.*, 2015), fibroblast-activating protein (FAP), vimentin (Gabbiani 2003; Liu *et al.*, 2006) and fibroblast specific protein-1 (FSP-1) (Sugimoto *et al.*, 2006).

Therefore, we performed qPCR assays using two most commonly up-regulated markers in CAFs (α -SMA and IL-6) for further confirmation that normal oral fibroblasts were activated and transformed to CAFs upon exposed to condition media from isolated cells. Our results revealed a high significantly up-regulation of both investigated markers (α -SMA and IL-6) when fibroblasts were incubated with conditioned media collected from isolated cells (early adherent and chemoresistant) for the both examined oral cancer cell lines (H357 and SCC4) in comparison to their levels exhibited by fibroblasts subjected to conditioned media of unsorted cells. These results are consistent with many reports that showed increased levels of expression of α -SMA by CAFs (Tuxhorn, Ayala and Rowley, 2001; Kunz-Schughart and Knuechel, 2002; Gabbiani, 2003; Huang *et al.*, 2010; Shintani *et al.*, 2016). In addition, several studies revealed up-regulation of IL-6 in CAFs which support our findings (Giannoni *et al.*, 2010; Vicent *et al.*, 2012; Osuala *et al.*, 2015; Yeh *et al.*, 2015; Lee *et al.*, 2015; Shintani, Fujiwara and Kimura, 2016). However, our finding showed significantly down-regulated levels of IL-6 expressed by the NOFs treated with TGF- β compared to that NOFs incubated with conditioned media from isolated cells (early adherent and chemoresistant) in all experiments. This could be attributed to variation in CAF phenotype which was identified by some studies that revealed despite these various

CAF phenotypes having the same common positive α -SMA protein expression, they possess divergent gene expression profiles (Mellone *et al.*, 2017).

Some literature suggests that cancer cells secrete cytokines and growth factors that mediate fibroblast activation into CAFs, which is considered an early and important event in tumourgenesis (Liotta and Kohn, 2001; Beacham and Cukierman, 2005; Amatangelo *et al.*, 2005). There are several stimuli and pathways reported to induce activation of quiescent fibroblasts into CAFs in tumour stroma. For instance, TGF- β , EGF, PDGF and FGF-2 secreted from injured epithelial cells as well as monocytes and macrophages provide support to the theory that tumours are non-healed wounds. Moreover, direct communication and contact via vascular cell adhesion molecule-1 (VCAM-1) or intercellular adhesion molecule-1 (ICAM-1) between fibroblasts and leukocytes leads to activation of fibroblasts into CAFs. Furthermore, reactive oxygen species, alteration in composition of ECM and complement factor C1 were reported to contribute to the activation of fibroblasts to CAFs (Kalluri and Zeisberg, 2006). Many studies consider TGF- β as the main mediator in altering the stromal responses of the tumour microenvironment in early stromagenesis through activating of fibroblasts to CAFs (Wever *et al.*, 2008; Sridhara *et al.*, 2013; Rao *et al.*, 2014). Such processes are maintained as a result of continuous TGF- β secretion by cancer cells and maintaining of increased levels of TGF- β receptors in CAFs (Wever and Mareel, 2003). In addition to the role of TGF- β in activation of CAFs, it plays a significant role with other factors such as PDGF and tumour necrosis factor (TNF- α) in maintaining CAFs within the tumour microenvironment (Orimo *et al.*, 2006; Shimoda, Mellody and Orimo, 2010). Moreover, literature reports additional roles of TGF- β in tumourgenesis, such as induction of EMT in CSCs and chemoattraction of infiltrating macrophages into the stroma of tumours (Wang *et al.*, 2007). However, increasing evidence on several types of tumours indicated that IL-6 induced transdifferentiation of fibroblasts to CAFs (Paland *et al.*, 2009; Giannoni *et al.*, 2010; Sanz-moreno *et al.*, 2011; Doldi *et al.*, 2015). Paland *et al.*, (2009) and Sanz-moreno *et al.*, (2011) revealed that activation of fibroblasts mediated by IL-6 has been through the IL-6/STAT3 pathway. Giannoni *et al.*, (2010) found in their study on prostate cancer that cancer cells released IL-6 which induced activation of normal prostate fibroblasts and the resultant CAFs in turn secreted IL-6, which mediated EMT in cancer cells that enhanced tumour invasiveness. In addition, Schauer *et al.*, (2011) showed that ovarian

cancer cells activated normal fibroblasts in tumour stroma through secretion of IL-6, IL-8, and IL-1 β cytokines. Furthermore, some reports found that aggressive growth of tumours was mediated through the TGF- β – IL-6 axis due to raised secretion of IL-6 and TGF- β in several types of cells as a result of IL-6 signalling (Aoki *et al.*, 2006; Lockett-Chastain and Gallucci, 2009). In turn, CAFs in activated stroma induce EMT in cancer cells through production of IL-6 (Orimo *et al.*, 2006; Shimoda, Mellody and Orimo, 2010). Moreover, Yamada *et al.*, (2013) found in their study a vicious cycle was formed due to the crosstalk between TGF- β and IL-6, which enhances tumour growth. Whereas, Doldi *et al.*, (2015) showed a close relation between TGF- β and IL-6 signalling and that either one of them can induce activation of fibroblasts in the stroma of prostate cancer.

We could interpret the activation of normal oral fibroblast subjected to conditioned media of isolated cells into CAFs using several explanations. Lygoe and her colleagues (2007) found in their study on oral and dermal fibroblasts that integrin pathways specifically $\alpha 5\beta 1$ (fibronectin receptor) and $\alpha v\beta 6$ (vitronectin receptor) regulate and induce TGF- $\beta 1$. Moreover, they showed when these integrin receptors were functionally blocked this lead to down-regulation of TGF- $\beta 1$ and subsequent inhibition of activation and differentiation of oral and dermal fibroblast into myofibroblasts. Our findings in chapter (4) from both FACs and qPCR analysis revealed that both isolated population of cells (early adherent and chemoresistant) from the both tested oral cancer cell lines (H357 and SCC4) expressed significantly high levels of integrin- $\beta 1$ (CD29) as well as up-regulated levels of integrin- $\beta 1$ gene expression compared to unsorted cells. Therefore, one of the mechanisms that might explain the activation of fibroblasts incubated with condition media of early adherent and chemoresistant cells could be attributed to their high expression of integrin- $\beta 1$ receptors that may result in higher production of TGF- $\beta 1$ which in turn increases activation of normal oral fibroblasts into CAFs. On the other hand, integrin- $\beta 1$ receptors mediate costimulatory signals that required for persistent inflammatory cytokine gene expression and subsequent production of inflammatory cytokines (Miyake *et al.*, 1993). Several studies reported that some inflammatory cytokines are secreted by cancer cells induce the activation of stromal fibroblasts into CAFs (Paland *et al.*, 2009; Giannoni *et al.*, 2010; Sanz-moreno *et al.*, 2011; Doldi *et al.*, 2015). Therefore, we could suggest another mechanism for activation NOFs exposed to

conditioned media from isolated cells is due to overproduction of proinflammatory cytokines such as IL-6, IL-8, and IL-1 β by isolated cells compared to that by unsorted cells (Schauer *et al.*, 2011).

Shintani and his colleagues (2016) conducted a study on lung cancer and they found that normal lung fibroblasts incubated with condition media of cancer cells treated with cisplatin showed an increase activation of these fibroblasts into CAFs compared to the normal fibroblasts that subjected to conditioned media from untreated cancer cells. Furthermore, they revealed that addition of a TGF- β 1 inhibitor to the conditioned media of cisplatin treated cells reduced the activation of normal fibroblasts into CAFs. Therefore, they inferred that treatment with cisplatin increases secretion of TGF- β 1 in cancer cells that could result in increased activation and differentiation of normal fibroblasts. This could explain our findings that normal oral fibroblasts incubated with conditioned media collected from chemoresistant cells were activated and expressed intense α -SMA staining as well as upregulated CAF markers.

Recent studies indicate the emerging and increasing role of cancer cell derived exosomes in triggering differentiation of normal fibroblasts into CAFs and activation of the tumour stroma. Webber and his colleagues (2010) revealed that normal fibroblasts differentiated into their fully activated phenotype (myofibroblasts) through the TGF- β 1 signalling pathway initiated by TGF- β 1 delivered from cancer-derived exosomes. They found that the myofibroblast phenotype resulting from activation by exosomal TGF- β 1 resembled but was not identical to that obtained through activation initiated by soluble TGF- β 1. Other studies attributed the effects of cancer-derived exosomes in the activation of normal stromal fibroblasts to some miRNAs that were transmitted through these exosomes. For instance, Pang *et al.*, (2015) reported that miR-155 delivered from pancreatic cancer-derived exosomes initiated transdifferentiation of normal murine pancreatic fibroblasts to CAFs. A similar study was conducted on ovarian cancer by Mitra *et al.*, (2012) and found that reprogramming of normal fibroblasts into CAFs was due to the effects of three miRNAs (miR31-miR-214 and miR-155) delivered by EVs secreted from cancer cells. Our data in chapter (4) demonstrated that the OSCC cells isolated by the two isolation methods (rapid adhesion and chemoresistance) secreted significantly elevated levels of EVs compared to unsorted cells. Therefore, we could attribute the activation of normal oral fibroblast subjected to conditioned media from isolated cells

(early adherent and chemoresistant) to the high concentrations of EVs secreted from these cells in their conditioned media in comparison to that of unsorted cells.

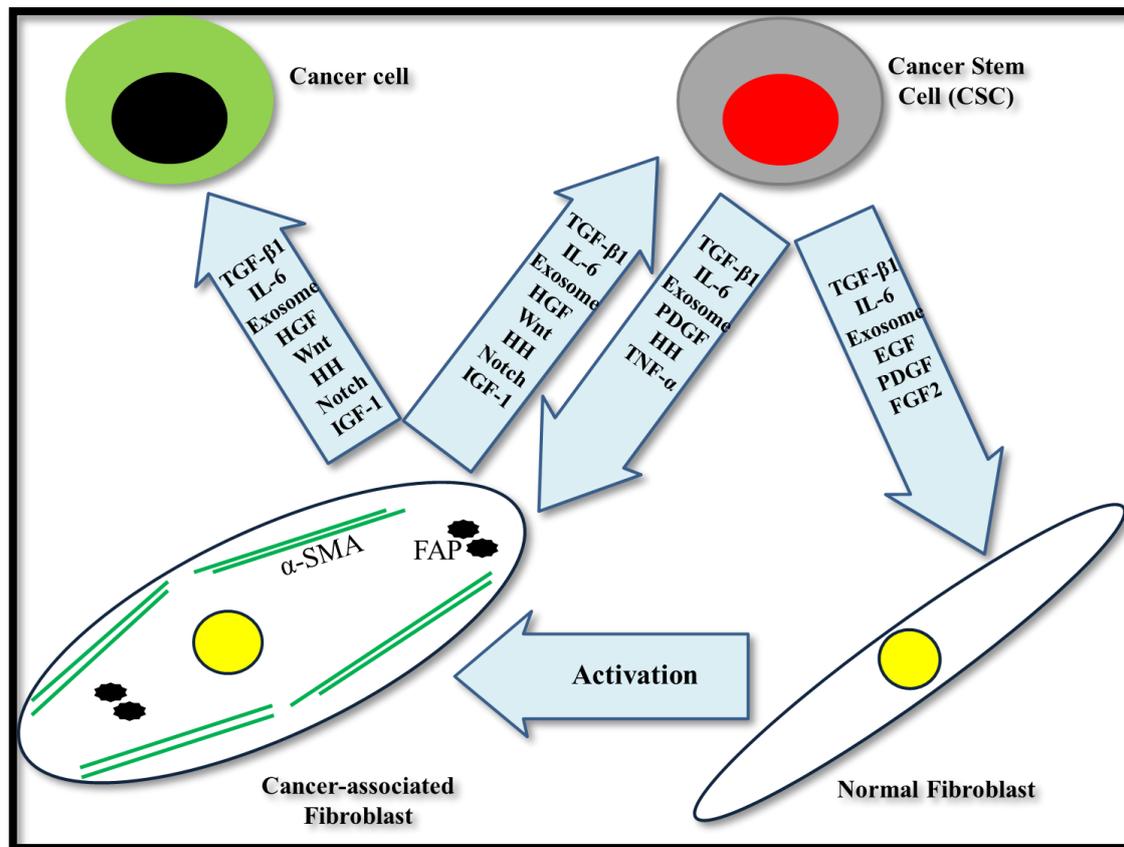


Figure 5.9: Activation of normal oral fibroblasts into cancer associated fibroblasts, and crosstalk between cancer cells and cancer associated fibroblasts within tumour stroma. TGF- β : transforming growth factor, IL-6: interleukin 6, EGF: endothelial growth factor, PDGF: platelet derived growth factor, FGF2: fibroblast growth factor 2, HH: Hedgehog ligand, TNF- α : tumour necrosis factor, HGF: hepatocyte growth factor, Wnt: Wnt ligand, Notch ligand, IGF-1: insulin-growth factor-1, α -SMA: smooth muscle actin fibres, FAP: fibroblasts activating protein.

5.6 Conclusions

We can deduce that NOFs are activated by factors in the conditioned medium secreted by isolated stem cells (early adherent and/or chemoresistant) leading to significantly higher expression of both α -SMA and IL-6 genes compared to the unsorted cells. However, the identity of activating factor(s) has not been identified during this study and further investigation is needed in the future to characterise them.

CHAPTER 6

***IN VIVO* EXPRESSION OF CSC
& FIBROBLASTS ACTIVATION
MARKERS IN OSCC**

6.1 Introduction

Oral cancer is classified as one of top ten malignancies worldwide with more than 300,000 new cases diagnosed each year (Ferlay *et al.*, 2015). Oral squamous cell carcinoma is considered the most common type of oral cancer and represents more than 90% of oral cancer cases (Choi and Myers, 2008). Although, recent reports indicate an overall reduction in mortality rate of cancer due to advances in treatment, the high mortality and low survival rates of oral squamous cell carcinoma remain unchanged because of its poor prognosis due to the high rates of local recurrence and distant metastasis (Michael *et al.*, 2008).

Recent studies proposed that initiation, development as well as the recurrence of tumours can be attributed to cancer stem cells (Oliveira, Jeffrey and Ribeiro-Silva, 2010; Papagerakis *et al.*, 2014). These cells form a small subpopulation of cells within tumour that possess unique characteristics such as self-renewal, ability of differentiation into diverse types of cells and resistance of anticancer therapies (Reya *et al.*, 2001; Nguyen *et al.*, 2012; Yua *et al.*, 2013). CSCs have been identified and characterised in various tumour types through expression of a variety of specific cell surface markers (Al-Hajj *et al.*, 2003; Mannelli and Gallo, 2011; Yua *et al.*, 2013; Routray and Mohanty, 2014). There is increasing evidence that CD24 and CD44 are key markers of CSCs in oral squamous cell carcinoma (Han *et al.*, 2014). Both CD24 and CD44 are cell surface molecules that play different roles in cancer. CD24 is glycosylated mucin protein, while CD44 is a glycoprotein (Kristiansen *et al.*, 2003; Collins *et al.*, 2005; Prince *et al.*, 2007; Sagiv *et al.*, 2008)

It has been reported that activation of host stromal cells by cancer cells is a crucial step in solid cancer progression. Despite this, the mechanisms of activation of the tumour stroma is not fully understood. In addition, the scientific literature indicates that cancer associated fibroblasts (CAFs) are the most prominent hallmark of the activated stroma. These CAFs are characterised by their expression of alpha smooth muscle actin (α -SMA) (Barth *et al.*, 2004; Kojc *et al.* 2005).

In this chapter we performed immunohistochemical analysis of samples of oral squamous cell carcinoma to examine the expression of CSC markers (CD24 and CD44) as well as a CAFs marker (α -SMA) to investigate if there is a correlation

between their expression and therefore whether there is a link between presence of CSCs and activated stroma.

6.2 The aims of the chapter

To investigate the concomitant expression of CSC specific markers (CD24 and CD44) with the a CAFs specific marker (α -SMA) in oral squamous cell carcinoma using immunohistochemistry.

6.3 Materials and methods

- The study sample consisted slides from 10 patients with squamous cell carcinoma in Sheffield. All the cases have a moderate grade of differentiation with various size and depth. Although all the cases are metastatic (invaded one or more of regional lymph nodes), some of them have extracellular spread (spread through the full thickness of the capsule of lymph node). Moreover, they vary in the degree of host response (represented by lymphocytes infiltrating through margin of tumour) ranging from poor to moderate and strong. Full clinical and histopathological data of patients and tumours in the selected study samples illustrated in table 6.1.
- Immunohistochemistry staining method (section 2.15).
- Quantification method (section 2.15).

Table 6.1: Clinical and histopathological data of patients and tumours in the selected study sample

case No.	Age	Gender	Tumour grade	Size mm	Depth	Front	Host response	Perineural	Vascular	Margin	Metastasis	Extracapsular spread
1	88	Female	Moderate	34	10	Discohesive	Moderate	No	No	Close	Yes	Yes
2	68	Male	Moderate	18	5.5	Cohesive	Poor	No	No	Clear	Yes	No
3	63	Male	Moderate	30	23	Discohesive	Moderate	No	Yes	Involved	Yes	No
4	79	Male	Moderate	30	17	Discohesive	Poor	Yes	No	Close	Yes	No
5	55	Female	Moderate	12	1.5	Cohesive	Poor	No	No	Involved	Yes	No
6	42	Female	Moderate	30	12.5	Discohesive	Poor	Yes	No	Close	Yes	Yes
7	70	Female	Moderate	49	11.5	Discohesive	Moderate	No	Yes	Close	Yes	Yes
8	71	Male	Moderate	62	20	Cohesive	Moderate	No	No	Involved	Yes	Yes
9	91	Male	Moderate	14	6	Discohesive	Strong	No	No	Clear	Yes	Yes
10	72	Female	Moderate	50	8	Discohesive	Moderate	Yes	No	Close	Yes	Yes

6.4 Results

6.4.1 Assessment of the correlation of immunohistochemical expression of CSC and CAF markers in OSCC.

Immunohistochemical analysis was conducted on slides from 10 cases of various oral squamous cell carcinoma (table 6.1) using human anti-CD24, anti-CD44 and anti-alpha smooth muscle actin (α -SMA) antibodies to investigate the expression of CSC markers (CD24 and CD44) as well as a CAF marker (α -SMA). The immunohistochemical stained slide were quantified and an average of stain intensity of each tested marker was calculated for all examined cases, as illustrated in table 6.2.

Table 6.2: Immunohistochemical stain intensity averages for CSC markers (CD24 and CD44) and the CAF marker (α -SMA) for each case of oral squamous cell carcinoma.

Case number	Marker stain intensity (pixels)		
	CD24	CD44	α -SMA
1	70.3	53.6	34.1
2	56.9	53.7	30.7
4	14.3	23.7	8.4
5	48.7	51.2	29.9
6	24.9	47.9	22.4
7	17.6	15.5	15.6
8	10.2	24.3	16.2
9	31.2	44.3	26.6
10	25.5	28.9	18.2

Our data showed an increased intensity of α -SMA stain in the cases that exhibited concomitant high intensities of both CD24 and CD44 such as case number 1, 2 and 5, as shown in table 6.2. The microscopic photos of these cases show an abundance of α -SMA stain in the stroma concentrated close to the basal cell layer of tumour epithelium at the tumour invasive front. In addition, intense α -SMA staining can be seen directly adjacent and surrounding islands of the epithelial tumour cells in the stroma, distributed either in a network or reticular pattern that demarcates the tumour invasive front, as illustrated in figures 6.4, 6.5 and 6.6.

Furthermore, our results indicated highly significant positive correlations between the expressions of both CD24 and CD44 stain in tumours and the expression of α -SMA stain in the stroma. Pearson's coefficient of the correlation between CD24 and α -SMA was $r = 0.91$ and P value = 0.0006, whereas for the correlation of CD44 with α -SMA $r = 0.89$ and P value = 0.001, figures 6.1 and 6.2.

Similarly, expression of CSC markers CD24 and CD44 in tumours of the examined OSCC cases revealed a strong positive correlation between them ($r = 0.83$, $P = 0.005$), figure 6.3.

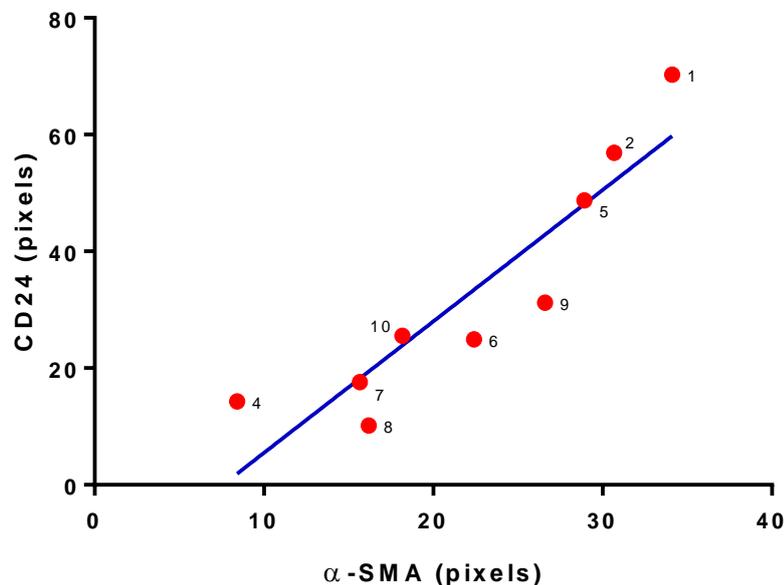


Figure 6.1: Scatter plot showing the significant positive correlation between expression of CD24 stem cell marker in the tumour and expression of a CAF marker (α -SMA) in the tumour stroma, ($r = 0.91$, $P = 0.0006$).

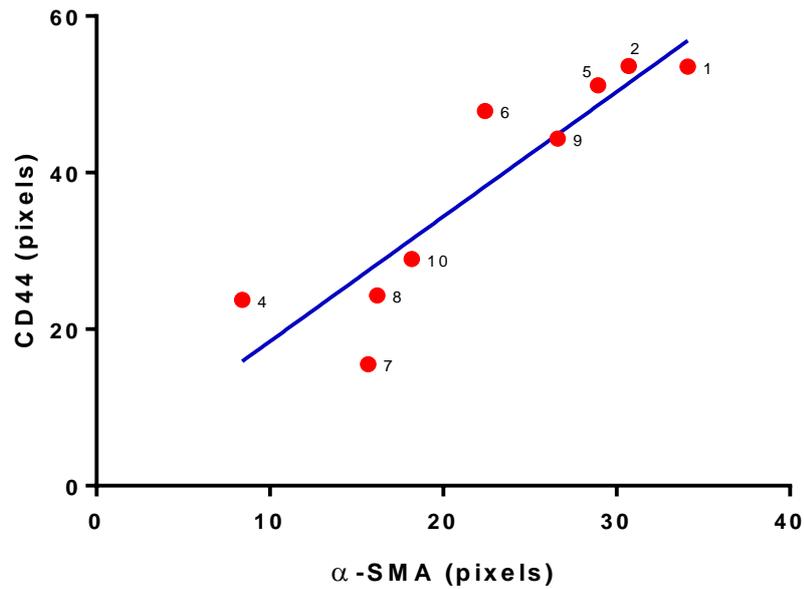


Figure 6.2: Scatter plot showing the significant positive correlation between the expression of CD44 stem cell marker in the tumour and expression of a CAF marker (α -SMA) in the tumour stroma, ($r = 0.89$, $P = 0.001$).

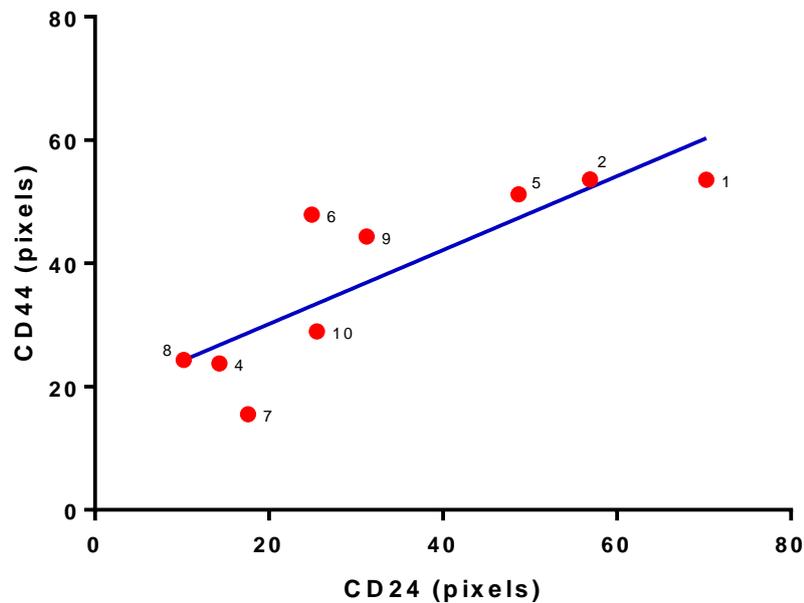


Figure 6.3: Scatter plot showing the significant positive correlation between the expression of the stem cell markers CD24 and CD44 in tumour sections, ($r = 0.83$, $P = 0.005$).

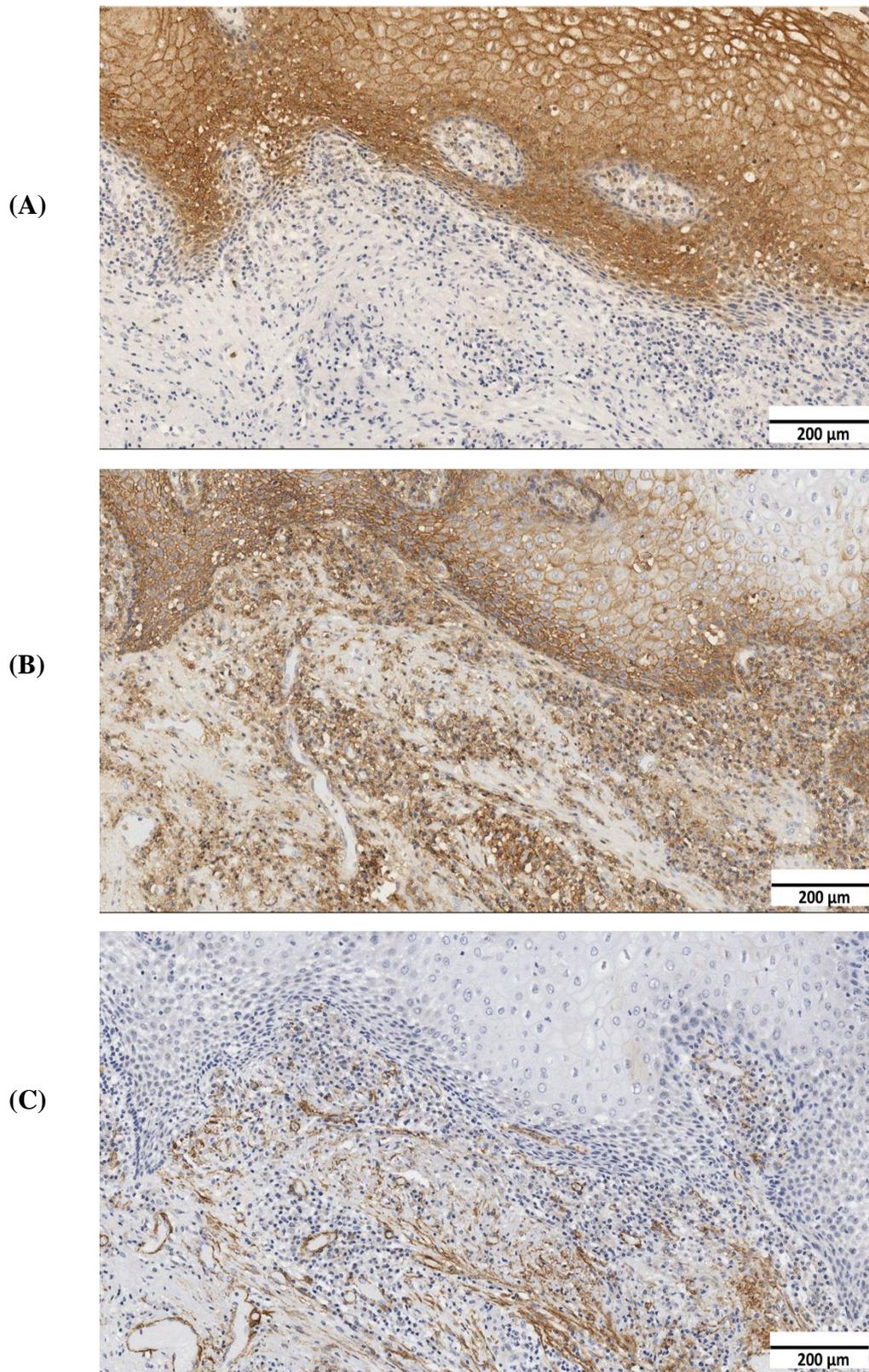


Figure 6.4: Representative photomicrographs of case number 1. Primary oral squamous cell carcinoma invading the underlying connective tissue. (A) CD24 staining is seen throughout the tumour epithelium (B) CD44 staining is seen throughout the tumour epithelium. (C) α -SMA staining is seen throughout stroma at the invasive front of the tumour. (Magnification: 100 \times). The brown stain indicates the expression of markers.

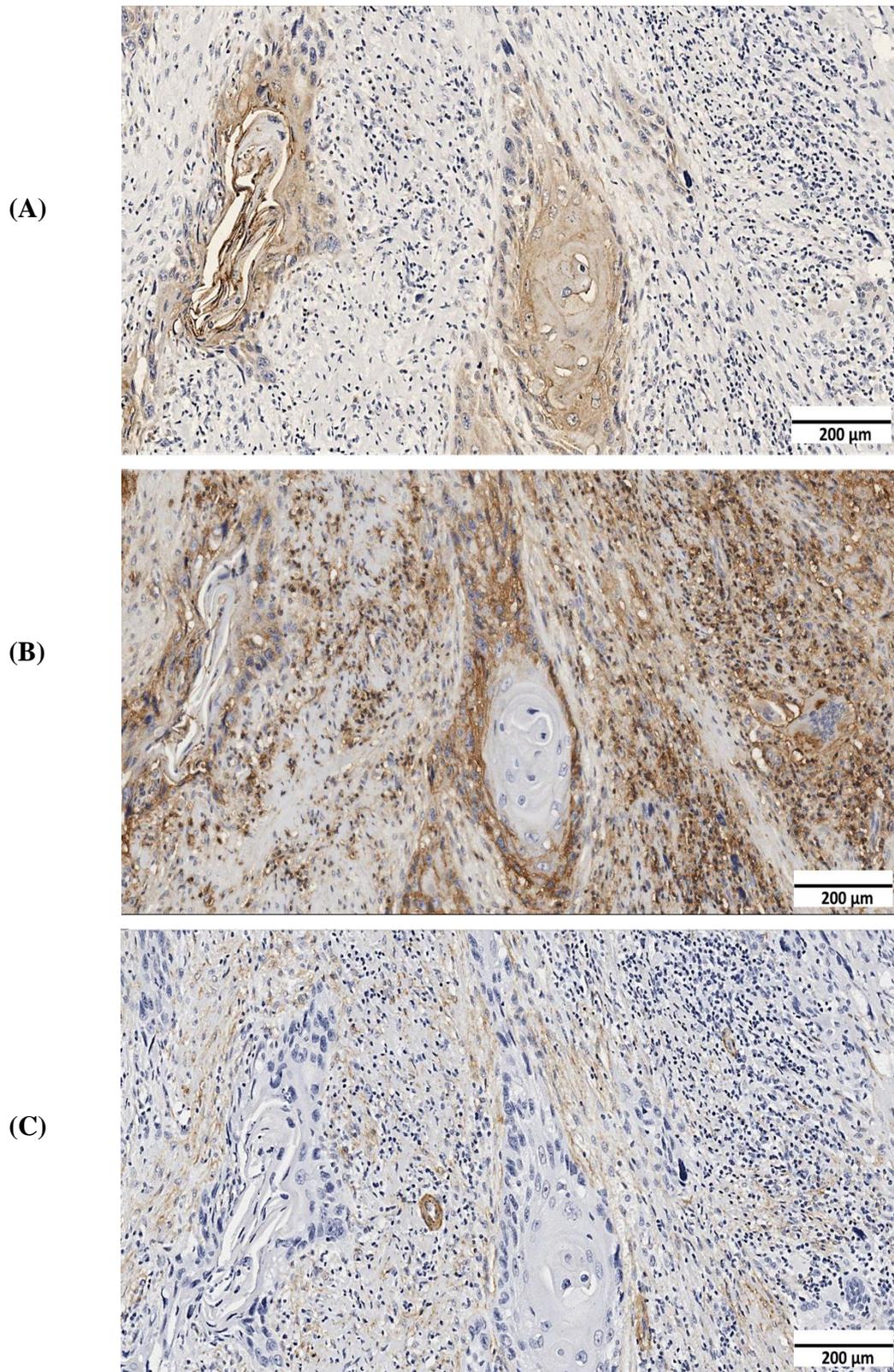


Figure 6.5: Representative photomicrographs of case number 2. Primary oral squamous cell carcinoma invading the underlying connective tissue. (A) CD24 staining is seen throughout the tumour epithelial islands. (B) CD44 staining is seen throughout the tumour epithelial islands. (C) α -SMA staining is seen throughout the stroma at the invasive front of the tumour surrounding tumour epithelial islands. (Magnification: 100 \times). The brown stain indicates the expression of markers.

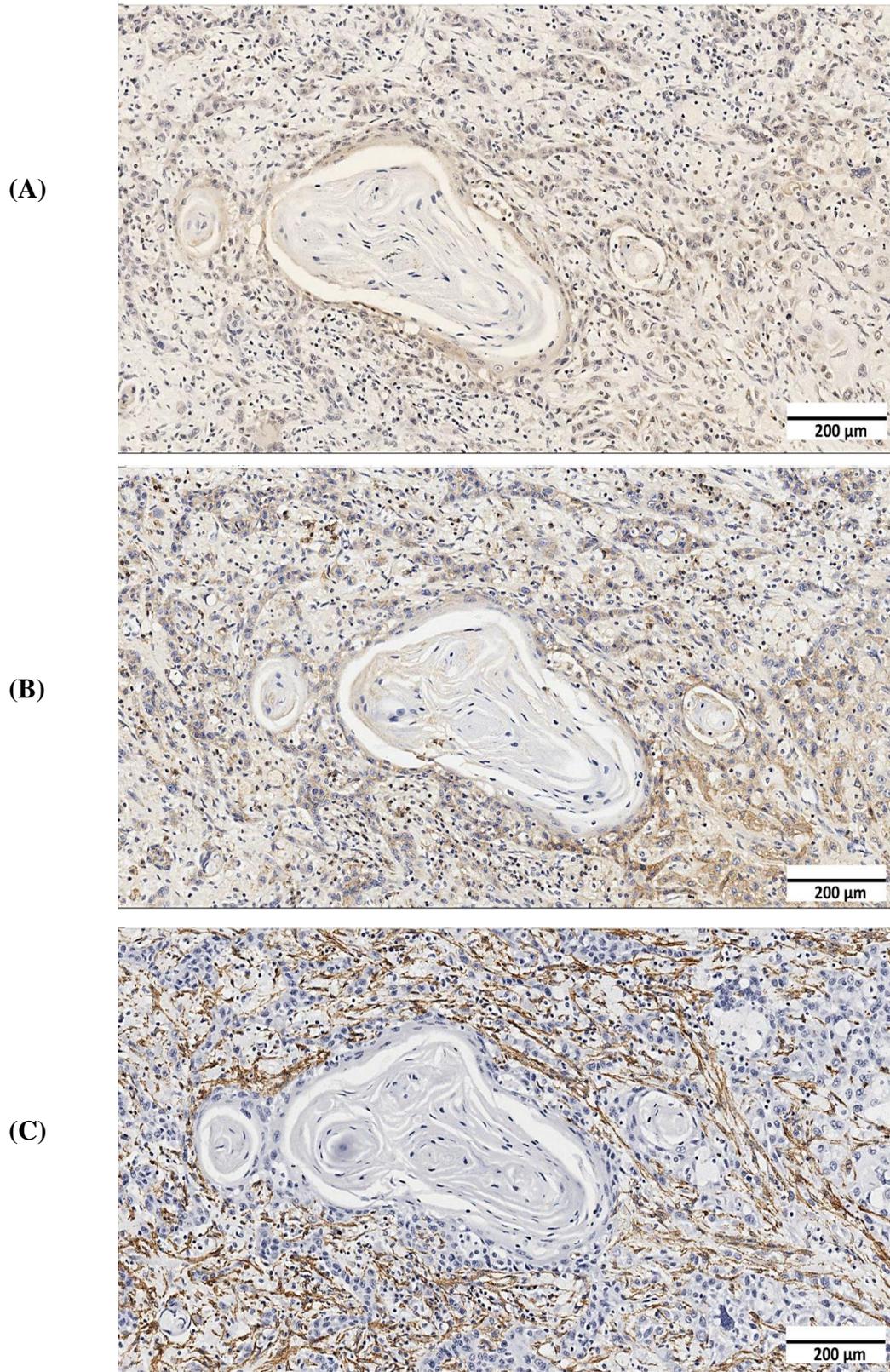


Figure 6.6: Representative photomicrographs of case number 5. Primary oral squamous cell carcinoma invading the underlying connective tissue. (A) CD24 staining is seen throughout the tumour epithelial islands. (B) CD44 staining is seen throughout the tumour epithelial islands. (C) α -SMA staining distributed throughout the stroma at the tumour invasive front surrounding tumour epithelial islands. (Magnification: 100 \times). The brown stain indicates the expression of markers.

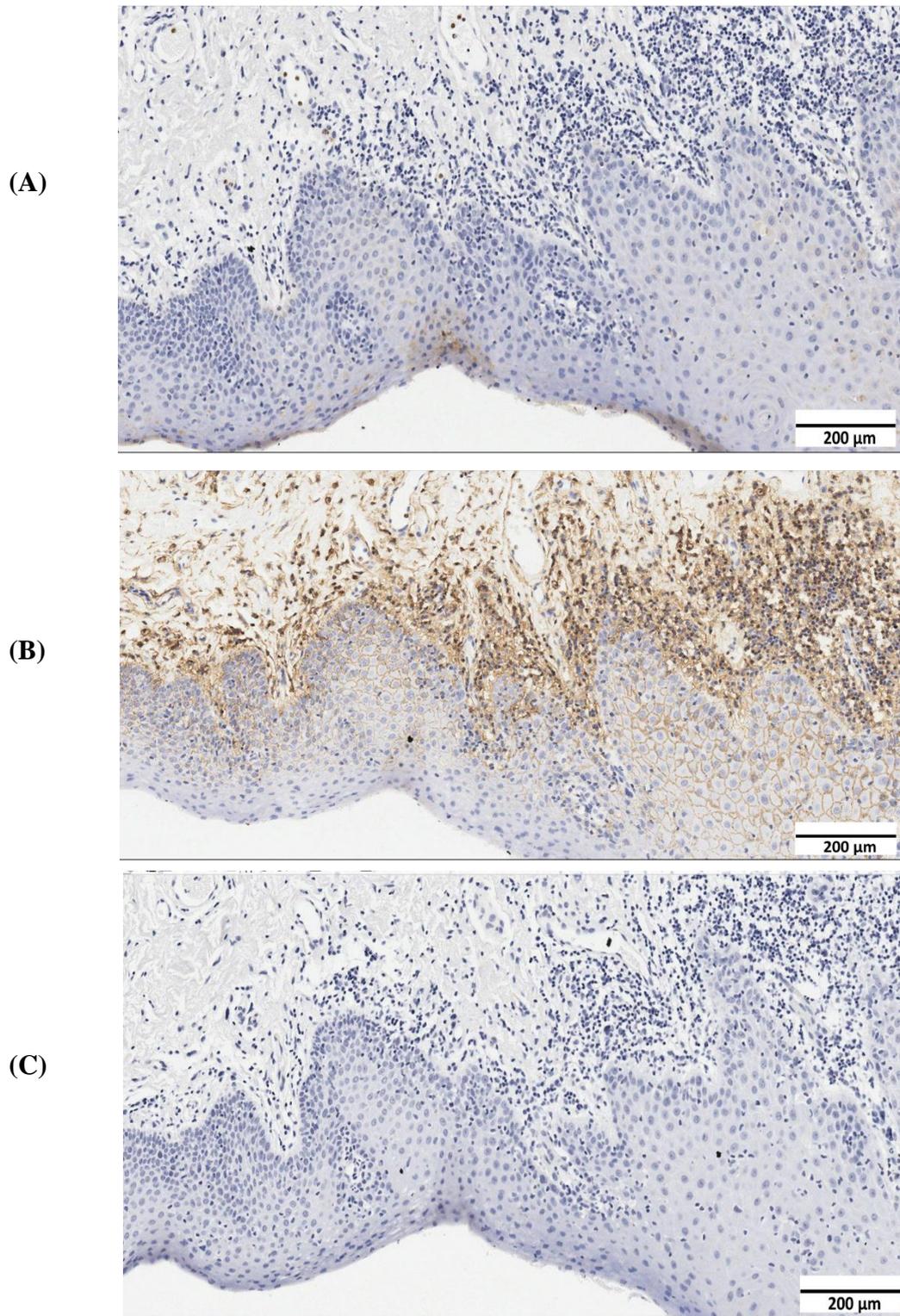


Figure 6.7: Representative photomicrographs of case number 4. Primary oral squamous cell carcinoma invading the underlying connective tissue. (A) CD24 very low staining is seen throughout the tumour epithelium. (B) CD44 low staining is seen throughout the tumour epithelium. (C) No noticeable α -SMA staining in the stroma. (Magnification: 100 \times). The brown stain indicates the expression of markers.

6.5 Discussion

The increasing importance of CSCs in the development and progression of many cancer types has been proposed recently (O'Connor *et al.*, 2014; Kreso and Dick 2014; Borah *et al.*, 2015). However, little is known about the behaviour and biology of CSC in oral squamous cell carcinoma. Currently, CSC studies are considered a promising area in OSCC oncology that might result in better understanding of tumourgenesis (Oliveira *et al.*, 2011).

It has been established that stem cell surface markers can be used to identify CSCs in various tumours (Singh *et al.*, 2003; Kristiansen *et al.*, 2003; Collins *et al.*, 2005; Ricci-Vitiani *et al.*, 2007). Furthermore, several studies reported using immunostaining analysis of the expression of stem markers as a potent method to evaluate the existence of CSC phenotype in some tumours including OSCC (Lim and Oh, 2005; Graziano *et al.*, 2008). We used the co-expression of CD24 and CD44 stem cell markers for immunohistochemical analysis as an indicator to evaluate the existence of CSCs in OSCC. A number of studies highlighted that cells with co-expression of CD24 and CD44 were confirmed as CSCs in different types of cancer (Fang *et al.*, 2005; Li *et al.*, 2007; Ricci-Vitiani *et al.*, 2007), and including head and neck squamous cell carcinoma (Han *et al.*, 2009; Han *et al.*, 2014). In addition, our findings in chapter (4) showed increased expression of both CD24 and CD44 markers in CSCs isolated from oral cancer cell lines using functional assays (early adhesion and chemoresistance).

CD44 is a trans-membrane glycoprotein receptor for hyaluronate. Normally, it coordinates cell-cell adhesion, motility of cells, activation of lymphocyte and cell migration (Shtivelma and Bishop, 1991; De Marzo *et al.*, 1998). Moreover, it plays a key role in cellular interaction with hyaluronate, osteopontin, serglycin and ECM proteins such as fibronectin, collagen I and IV. On the other hand, it has been reported that CD44 is a pivotal CSCs marker which overexpressed in most of solid tumours (Assimakopoulos *et al.*, 2002). Furthermore, oral squamous cell carcinoma is considered as the second epithelial tumour type after breast cancer in which CD44 is the most frequent marker expressed by the CSC population (Hocwald *et al.*, 2001).

CD24 is a glycosated cell surface protein for P selectin receptor (the adhesion receptor of activated endothelial cells and platelets) (Weichert *et al.*, 2005; Lim, 2005). Normally, CD24 is expressed by keratinocytes, regenerating muscles, renal tubules and the developing pancreas and brain (Weichert *et al.*, 2005), as well as being a B cell marker (Weichert *et al.*, 2005; Lim, 2005). Previous studies have indicated that CD24 could be a key marker of CSCs in various cancers including oral squamous cell carcinoma (Han *et al.*, 2009; Han *et al.*, 2014). It has been suggested that the CD24 is a factor involved in regulation of cell adhesion, proliferation and apoptosis (Weichert *et al.*, 2005). Therefore, down- regulation of CD24 leads to inhibition of tumour cell proliferation and induction of their apoptosis, whilst up- regulation results in increased of tumour growth and enhancement of metastasis due to its activation of P selectin receptors on platelets and activated endothelial cells (Lim and Oh, 2005; Sagiv *et al.*, 2008).

Our results revealed a significant positive correlation between the expression of CD24 and CD44 markers in the study samples of oral squamous cell carcinoma. These results are consistent with the findings from other immunohistochemistry studies of CSCs in oral squamous cell carcinoma which reported increased levels of expression of both CD24 and CD44 (Han *et al.*, 2009; Oliveira *et al.*, 2011; Han *et al.*, 2014). However, there are no previous reports correlating expression of these two CSC markers.

Oral squamous cell carcinoma like other solid tumour types is described as a composite of cells due to the presence of cancer cells as well as various types of stromal cells such as fibroblast, inflammatory and endothelial cells. These cells interact in concert leading to tumour progression, local invasion and distant metastasis (de Vicente *et al.*, 2005). Therefore, there is coordination between transformation of normal epithelial cells into cancer cells and activation of normal stromal cells into cancer associated (Beacham and Cukierman, 2005; Amatangelo *et al.*, 2005). So, alteration and remodelling of stroma is initiated by cancer cells, whereas stromagenesis is a process induced by activated stroma (Beacham and Cukierman, 2005). The most distinctive feature of the activated stroma is the activation of normal stromal fibroblasts into myofibroblasts or CAFs (Giatromanolaki, Sivridis and Koukourakis, 2007). These cells show a phenotype between smooth muscle cells and fibroblasts that is characterized by the expression of alpha smooth muscle actin fibres

(α -SMA) (Desmouliere, Chaponnier and Gabbiani, 2005). It is evident that α -SMA immunostaining is not present in normal oral mucosa and premalignant oral lesions except by smooth muscle cells lining the blood vessel walls (Kellermann *et al.*, 2007).

Therefore, we selected α -SMA antibody in our immunohistochemical analysis to evaluate the existence of CAFs in the stroma of oral squamous cell carcinoma.

Our findings demonstrated significant positive correlation between the expression of both CSC markers CD24 and CD44 in the tumour and the expression of α -SMA in stroma. This correlation has not been previously reported. Although, we have not investigated a potential mechanism for this link, it agrees with the data obtained from previous chapter (5). These data that found activation of normal oral fibroblasts into CAFs upon incubation with conditioned media of early adherent and chemoresistant cells (CSCs) isolated from OSCC cell lines. However, several immunostaining studies conducted on OSCC indicate that epithelial cancer cells induced the activation of stroma and increased expression of stromal α -SMA (Etemad-Moghadam *et al.*, 2009; Prasad *et al.*, 2016). Furthermore, Marsh and his colleagues (2011) highlighted in their study the strong positive correlation between the high stromal α -SMA immunostaining expression with the increased aggressiveness and the mortality rate in OSCC patients regardless the stage of the disease and they consider stromal α -SMA rather than other tumour features as a crucial prognostic factor in OSCC. Kellermann *et al.*, (2007) found a significant positive link between the increased immunohistochemical expression of α -SMA in the tumour stroma and the increasing invasiveness and subsequent poor prognosis of OSCC. Similarly, many studies correlated the elevated concomitant immunostaining expression of CD24 and CD44 stem cell markers in oral squamous cell carcinoma with clinicopathological increasing of tumour progression, aggressiveness, invasion and metastasis (Han *et al.*, 2009; Oliveira *et al.*, 2011; Koukourakis *et al.*, 2012; AbdulMajeed, Dalley and Farah, 2013; de Moraes *et al.*, 2017). Han *et al.*, (2009) reported CD24 and CD44 immunostaining expression as important diagnostic markers for OSCC. Koukourakis *et al.*, (2012) revealed immunostaining overexpression of CSCs markers CD44, CD24 and CD29 correlated with the increased growth rate and resistance to radiotherapy in head and neck squamous cell carcinoma. AbdulMajeed, Dalley and Farah, (2013) considered that increased immunostaining expression of CD24 a potent diagnostic marker for detection of oral epithelial dysplasia and OSCC. de Moraes *et al.*, (2017)

established a correlation between immunohistochemical overexpression of CSC markers CD24, CD44 and CD29 with the poor overall survival outcomes in patients with OSCC. Whereas, in our study we could not correlate the co-expression of CD24 and CD44 with clinicopathological features of selected OSCC samples because of our small sample size.

There are many quantification methods used to assess the immunostaining, ranging from manual to automated computer-assisted methods. Manual methods of immunohistochemical (IHC) staining assessment are performed with the naked eye using light microscopy. This method has drawbacks of high subjectivity and variability between individuals leading to errors as well as being time consuming (Schüffler *et al.*, 2013). The automated computer-assisted methods are developed software programs used to analyse the IHC staining. The scoring of IHC staining analysis is calculated on the basis of modern cellular imaging system (Mane, Kale and Belaldavar, 2017). Many software programs were developed to use for IHC analysis such as: VORSTAIN (Guillaud *et al.*, 1997), CellProfiler (Carpenter *et al.*, 2006), KNIME (Berthold *et al.*, 2007), Vaa3D (Peng *et al.*, 2010), BioImageXD, Icy (de Chaumont *et al.*, 2011), AQU Analysis (Klimowicz *et al.*, 2012), ImageJ (Schindelin *et al.*, 2012), TMARKER (Schüffler *et al.*, 2013), Multiplex IHC and Multispectral Image Analysis (Oguejiofor *et al.*, 2015). However, most of these software are expensive and need additional hardware attachments either for capturing images or for analysis (Mane, Kale and Belaldavar, 2017). Therefore, we selected ImageJ software system to quantify our IHC staining analysis. It is characterised by its simple free software, easily installed and run on all operating systems and can be used by researcher with minimal computer skills as well as several plugins which are additional software components could be installed to increase its applications (Schindelin *et al.*, 2012). Furthermore, there is very minimal interobserver variability reported during using of ImageJ in IHC marker staining analysis that performed on formalin-fixed tissue (Mane, Kale and Belaldavar, 2017). ImageJ has been widely used in cancer research to investigate several diagnostic and prognostic markers in various cancer types (Kolenda *et al.*, 2011; Hirata *et al.*, 2013; Takemura *et al.*, 2014; Okabe *et al.*, 2014; Pietras *et al.*, 2014). In addition, many immunohistochemical analysis were conducted in recent oral squamous carcinoma research using ImageJ software. For instances: Sun *et al.* (2010) investigated the expression of CD44 and

ABGG2 markers in OSCC. Grimm et al (2012) examined the link between the co-expression of CD44 and ABCB5 with tumour progression and recurrence. Spiegelberg et al (2014) investigated CD44 variant expression in head and neck squamous cell carcinoma. Sawant et al (2016) investigated the expression of CD44, Oct4 and c-Myc prognostic factors in OSCC patients who received radio or chemotherapy. Finally, Ghuwalewala et al (2016) analysed the immunohistochemical expression of CD24 and CD44 CSCs markers in OSCC samples.

6.6 Conclusions

We could conclude that the correlation between CSC and fibroblast activation markers *in vivo* suggests that CSCs *in vivo* induce activation of the tumour stroma in oral squamous cell carcinoma and, as a consequence, drive of tumourgenesis.

CHAPTER 7

**GENERAL DISCUSSION,
CONCLUSIONS & FUTURE
PERSPECTIVES**

7.1 General discussion

The cancer stem cells (CSCs) hypothesis suggests the existence of a small population of undifferentiated cells within a tumour, characterised by unique intrinsic features such as their ability of self-renewal, unlimited proliferation and driving of tumourgenesis (Islam, Qiao, *et al.*, 2015). In addition, they are considered tumour initiating cells that have distinct genetic and epigenetic heterogeneity and ability of resistance to conventional chemo and radiotherapies leading to relapse of a tumour and later initiation of metastasis (Zhou *et al.*, 2009; Frank, Schatton and Frank, 2010; Islam, Qiao, *et al.*, 2015). Furthermore, it has been reported in several cancer types the existence of a clinical correlation between the aggressive nature of CSCs and increasing recurrence and metastasis of tumour as well as the overall poor patient outcome (Prince and Ailles, 2008). Therefore, there is an increased demand to find effective methods to isolate and identify CSC populations within different tumours including the head and neck squamous cell carcinoma in order to thoroughly analyse their biological behaviour and develop novel therapies to targeting and eradicate them (Zhou *et al.*, 2009; Beck and Blanpin, 2013).

It has been established that the existence of CSCs (which are immortal) is not limited to patient tumour samples, but their presence is also found and persisted under different conditions for years and decades in many cancer cell lines derived from a wide range of tumour types (Singh *et al.*, 2003; Al Hajj *et al.*, 2003; Kondo, Setoguchi and Taga, 2004; Patrawala *et al.*, 2005), including head and neck squamous cell carcinoma (Locke *et al.*, 2005; Mackenzie, 2006). Therefore, we used two oral cancer cell lines (H357 and SCC4) in our methodology to develop functional approaches to isolate and characterise CSCs in oral squamous cell carcinoma.

Isolation of a purified population of CSCs from tumours still a challenging issue, although it is a fundamental aim for most cancer stem cell studies. Many studies have been conducted to identify and isolate cancer stem cells from various tumours and most of them are dependent on the detection of several stem cell surface markers (antigens) using flow cytometry (Vermeulen *et al.*, 2012). In spite of FACS being considered the most common approach for CSCs isolation, several drawbacks have been reported. For instance, it is costly, good quality antibodies are required and the user should be well-trained and highly skilled (Walia and Elble, 2010). In addition,

there are several technical factors influencing the accuracy of results, such as: modifications of the cell phenotype upon antibody binding, subjectivity and inconsistencies of gate setting as well as cell preparation (type and concentration of the sorting cells) (Li *et al.*, 2005; Igarashi *et al.*, 2008). Chen *et al.*, (2014) reported that expression of CSC surface markers does not necessarily reflect the clinical manifestations or behaviours of cancer stem cells. Furthermore, several stem cell surface markers are expressed by non-CSCs as well as CSCs. Therefore, in our study we developed two functional assays to sort CSCs from oral cancer cell lines on the basis of two intrinsic characteristic features of CSCs which are the rapid adherence to ECM proteins and chemoresistance to conventional chemotherapy agents.

It is evident that the percentage of CSCs forms approximately 0.1-10% of all tumour cells within any tumour or cancer cell line (Deonarain, Kousparou and Epenetos, 2009). Both functional assays (adhesion and chemoresistance) developed in this study robustly and effectively yielded an average number of sorted cells from the both examined oral cancer cell lines (H357 and SCC4) of less than 10% of the total number of seeded unsorted cells.

Our study is considered the first study that isolated CSCs using two functional approaches and at the same time found a correlation between the two sorted cell populations since cells that were rapidly adherent to fibronectin from both examined cell lines were more chemoresistant to cisplatin and the chemoresistant cells showed enhanced rapid adherence to fibronectin. As a consequence, this significant correlation indicates that the two developed functional methods isolated the overlapping population of cells and suggests that the two methods sorted a highly purified population of CSCs.

The phenotype of isolated cells from the adhesion assay from the both oral cancer cell lines showed a stability and continuation of rapid adherence after incubation in culture for 48 hours. At the same time, the phenotype of chemoresistant cells from both oral cancer cell lines was stable with increased viability and growth rate following exposing to cisplatin treatment for the second time. The stability of CSCs phenotype is attributed mainly to tumour phenotypic equilibrium in which the various cell populations in a tumour tend to maintain their proportions overtime (Gupta *et al.*, 2011; Iliopoulos *et al.*, 2011; Zapperi and La Porta, 2012). This study is the first study

to investigate the stability of sorted early adherent cells phenotype after isolation and whilst some studies used other ECM protein (collagen IV) for sorting of CSCs they did not examine the stability of the rapid adherent phenotype afterwards (Lia, 2005; Ling *et al.*, 2014). However, some previous studies examined the stability of the chemoresistant cell phenotype by exposing them to chemotherapeutic drugs again following isolation and found that chemoresistant cells continue to be less sensitive with high viability (Bertolini *et al.*, 2009; Kim *et al.*, 2012), and the results of our study are consistent with them.

A tumour is defined as heterogeneous mass that is composed of a variety of cells which vary in their phenotypic and genetic characteristics, such as cellular morphology, proliferation rate, metabolism, metastatic potential and gene expression (Islam, Gopalan *et al.*, 2015). Therefore in any study, characterisation is considered an essential step to confirm the validity of isolated CSCs populations from a tumour. Characterisation includes investigating the stem cell characteristics of the sorted cells. Benchaour *et al.*, (2004) reported some stem cell criteria that should be present in any sorted cells population to validate them as CSCs. These criteria are: they represent a small population within tumour, have the ability of self-renewal and differentiation to non-tumorigenic cells as well as possess a distinctive gene profile and cell surface antigens. However, CSCs can be identified based on specific molecular, biological and functional features. For instance, differential and positive expression of stem cell surface markers and mRNA profile as well as functional properties such as colony formation, regeneration of tumour in immunodeficient animals, chemoresistance and aldehyde dehydrogenase activity (Milne *et al.*, 2009; Lianidou and Markou, 2011; Tirino *et al.*, 2012; Podberezin, Wen and Chang, 2012). Therefore in our study, we selected both molecular and functional assays for characterisation of the isolated cells from both functional methods (early adherent and chemoresistance) that we used in chapter (3) to ensure the presence of various 'stemness' features in the sorted cells.

It has been reported that expression of stem cell surface markers remains the most common assay for characterising cells with 'stemness' properties (Shah *et al.*, 2014). However, there is no universal marker identifying cancer stem cells and there may be tumour type specific variation of some markers. Moreover, it has been recommended that a combination of markers be used to get high specificity in characterising CSCs from a specific tumour (Karsten and Goletz, 2013). Therefore, we selected multiple

stem cell surface markers that were commonly reported in head and neck squamous cell carcinoma studies as markers for identifying CSCs in OSCC which are CD44, CD24 and CD29. Our results showed increased protein and gene expression of all these markers by the isolated cells (early adherent and chemoresistant) from both investigated oral cancer cell lines (H357 and SCC4). This strong correlation between stem associated genes and cell surface proteins of the sorted cells in our study indicates that these cells have the same pattern of gene and surface protein expression and support their 'stemness' characteristics. In addition, *in vitro* functional assays (proliferation and colony forming assays) performed during characterisation of sorted cells using the two isolation methods for both cancer cell lines used in this study confirmed their 'stemness' characteristics as the isolated cells expressed low growth rate and high clonogenic potential. Subsequently, the results of the both molecular and biological assays conducted for characterisation of the early adherent and chemoresistance cells established them as cancer stem cells.

Our results showed an increased ability of the sorted cells (CSCs) to secrete EVs compared to unsorted cells which may suggest a role for EVs as one of mechanisms of CSCs communication with other cancer and stromal cells within a tumour. In addition, it might support the role of CSCs as key players in modulation of tumour progression. Several molecules are transferred between various cells in the tumour microenvironment by EVs including: mRNA, microRNA, proteins and lipids (Hannafon and Ding, 2013). Evidence from the current literature suggests that CSC-derived EVs are transferred to surrounding stromal and non-cancer cells and may have a key role in the modulation of tumour progression (Hannafon and Ding, 2015). Gross *et al.*, (2012) and Lin, Wang and Zhao, (2013) established in their studies the presence of Wnt protein ligand in exosomes derived from human cancer cell lines that induce the Wnt signalling pathway in the recipient cells. In addition, several studies report that TGF- β proteins are transferred through cancer cells derived EVs. For instance, Cho *et al.*, (2012) and Chowdhury *et al.*, (2015) indicated in their studies that TGF- β released from cancer cells derived exosomes induces the TGF- β signalling pathway in mesenchymal stem cells with subsequent mediation of their differentiation to a myofibroblast like phenotype. Whereas, Webber *et al.*, (2015) showed in their study that TGF- β in cancer cell derived exosomes triggered differentiation of fibroblasts to myofibroblasts in the prostate tumour microenvironment. Moreover,

some studies reported the influence of microRNA carried by cancer cell derived exosomes. Abd Elmageed *et al.*, (2014) identified several oncogenic microRNAs in prostate cancer cell derived exosomes such as miR-155, miR-130b and miR-125b that cause reprogramming and transformation of adipose stem cells to CSCs. In addition, two studies conducted on prostate CSC revealed that microRNAs expressed in CSC derived exosomes significantly enhanced EMT and pre-metastatic niche formation and subsequent metastasis compared to that expressed in exosomes released by non-CSCs (Sánchez *et al.*, 2015; Rana and Malinowska, 2013). It has been suggested that cancer derived EVs play a role in cancer cells escaping from the immune response through different mechanisms (Naito *et al.*, 2017). Wieckowski *et al.*, (2009) and Szajnik *et al.*, (2010) indicated a role for exosomes released from cancer cells in the suppression of anticancer immunity through induction of apoptosis of CD8⁺ T cell and promotion expansion of regulatory T cells. De Vrij *et al.*, (2015) found that cancer cell derived EVs enhanced evasion of immunity via induction of differentiation of monocytes to immunosuppressive macrophages, while Chow *et al.*, (2014) show in their study that EVs modulate anticancer immunity through induction of pro-inflammatory cytokines such as TNF- α , CCL2 and IL-6 by activation of NF- κ B signalling following internalization of EVs into macrophages. In addition, literature suggests CSC derived exosomes influence cancer progression. Grange *et al.*, (2011) found that angiogenesis and premetastatic niche formation were triggered by renal CSC derived exosomes. Bourkoula *et al.*, (2014) and Bronisz *et al.*, (2014) reported in their studies on glioma CSC that glioblastoma aggressiveness and invasion were promoted by glioma CSC derived EVs. The effects of EV secretion are bidirectional in that CSC do not only release EVs but they also receive EVs from other surrounding stromal cells in the tumour microenvironment. For example, stromal cells support self-renewal of CSC through exosomal signalling (Wu *et al.*, 2014).

Previous studies have highlighted the pivotal role of cancer associated fibroblast (CAFs) in promoting and enhancing of tumour progression. Such cells secrete abundant ECM proteins and proteases and many soluble factors such as cytokines and growth factors into the tumour stroma. As a result, they stimulate proliferation of tumour cells and modulate immune response and resistance to anticancer therapies. In addition, they induce angiogenesis, invasion and metastasis (Kalluri and Zeisberg, 2006; Ishii *et al.*, 2016). Literature reports that the activation of normal fibroblasts

into CAFs in the tumour microenvironment is induced by signals from tumour cells (Elenbaas and Weinberg, 2001; Wever and Mareel, 2003; Kalluri and Zeisberg, 2006). Moreover, it has been established that the cross-talk between cancer and stromal cells in a tumour microenvironment plays a dynamic role in tumour progression (Patten *et al.*, 2008; Buggins *et al.*, 2010; Pepper *et al.*, 2011). Orimo and Weinberg, (2006) reported that the preferential residence of both CSCs and CAFs at the tumour-stroma interface leads to reciprocal support and interaction with each other. Therefore, in our study we investigated which population of tumour cells in particular activates the stromal fibroblasts into CAFs because of heterogeneity in the tumour cells population. Subsequently, we investigated in chapter (5) the effects of conditioned media collected from isolated cells (CSCs) on the normal oral fibroblasts. We found an intense expression of α -SMA fibres in the immunofluorescence analysis by the normal oral fibroblasts that were incubated in conditioned media from the CSCs (early adherent and chemoresistant). The resultant CAF phenotype was further confirmed by expression of significantly increased levels of CAF-associated genes (α -SMA and IL-6) that were revealed by qPCR analysis. Therefore, we concluded that CSCs in the tumour cell population induce the activation of the stromal fibroblasts into CAFs in the tumour microenvironment and as a consequence they drive the tumorigenesis process through their cross-talk with CAFs which in turn promote and enhance the stromagenesis, tumour growth, invasion and metastasis. Therefore, developing approaches to target and block the cross-talk and the communication between CSCs and CAFs could be an effective approach in anticancer therapy in the future.

It is well known that solid tumours including head and neck squamous cell carcinoma are identified as composite tumours, due to the existence of cancer cells and variety of stromal cells such as fibroblasts, inflammatory and endothelial cells within the tumour mass. Furthermore, there are dynamic interactions among these cellular elements of a tumour (de Vicente *et al.*, 2005). Due to our findings that showed the influence of CSCs conditioned media on the activation of stromal fibroblasts. We examined the *in vivo* correlation of CSC and CAFs in the tumour microenvironment of oral squamous cell carcinoma patient samples. This was performed by investigating of the immunohistochemical expression of two of most common reported CSC markers of head and neck squamous cell carcinoma which are CD24 and C44 and a marker of

activated fibroblasts (α -SMA). Our results revealed an *in vivo* positive correlation between the immunohistochemical expression of CSC markers (CD24 and CD44) in tumour and the CAFs marker (α -SMA) in the stroma which confirms our previous finding of *in vitro* activation of stromal fibroblasts by CSCs. Additionally, it suggests a mechanism by which CSC drive tumour progression.

The high mortality and recurrence rates of oral squamous cell carcinoma as well as the increasing importance for its early detection suggests more work is required on the basic understanding of disease progression. Our findings suggest the possibility of future studies using these CSC markers to develop new diagnostic and prognostic biomarkers which could improve the early detection and prevent recurrence of oral squamous cell carcinoma.

7.2 General conclusions

1. Rapid adhesion to fibronectin and chemoresistance to cisplatin are sensitive and effective functional methods to isolate CSC population in oral squamous cell carcinoma cell lines.
2. The increased chemoresistance of early adherent cells and the increased adherence of cisplatin-resistant cells suggest that these two methods are isolating phenotypically overlapping highly purified populations of CSCs.
3. The isolated phenotype (early adherent and/or chemoresistant) was stable at least 48 hours after isolation.
4. The sorted cells by the two functional methods (early adhesion and chemoresistance) from the both examined oral cancer cell lines exhibited molecular and functional CSC-like characteristics.
5. CSCs (early adherent and chemoresistance) secreted enhanced levels of EVs compared to other unsorted cancer cells.
6. Normal oral fibroblasts (NOFs) are activated into CAFs by the factors released from isolated stem cells (early adherent and/or chemoresistant).
7. As there is a positive correlation between CSC and activated fibroblasts *in vivo*, CSCs may drive tumourigenesis through the activation of stromal fibroblasts in the tumour microenvironment.

7.3 Future perspectives

1. Many studies report that xenotransplantation is the gold standard assay for characterizing of CSCs (Tirino *et al.*, 2012; Dobbin and Landen, 2014). Therefore, it would be important to investigate the tumourigenic potential of the isolated cells *in vivo* and examine their ability to initiate and generate tumours in immunodeficient animals. This could be performed through xenotransplantation after sorting CSCs using our developed functional methods (early adhesion and chemoresistance). Four groups of immunodeficient mice would be investigated for each oral cancer cell line. The first group would be injected with fresh early adherent cells after their isolation by adhesion assay from unsorted cells. The second group would be injected with chemoresistant cells following treating of the unsorted cells with 15 μ M cisplatin for 24 hours and after that the treatment is washed and incubated for 5 days with fresh growth medium. Whereas, the third group of mice are injected with isolated cells using both functional methods sequentially to increase the probability of getting high purified population of sorted CSCs. Therefore, the unsorted cells from both tested oral cancer cell lines are isolated firstly by rapid adherence to fibronectin 75 μ g/mL for 10 minutes. Then the early adherent cells are treated with cisplatin 15 μ M for 24 hours, then the drug is washed away with PBS and fresh growth medium is added for 5 days (the recovery period). Afterwards, the viable chemoresistant cells would be injected in immunodeficient mice. The last group of mice would be injected with unsorted cells.
2. Since we found in our study that CSC drive the tumourgeniesis through activation of stromal fibroblasts. It would be useful to investigate other mechanisms by which these sorted CSCs could affect the tumourgeniesis and cancer progression, such as the influence of CSCs on induction of EMT and acquiring an invasive phenotype in the other tumour cells. This could be studied by incubating unsorted cancer cells for 48 hours in the conditioned media collected from sorted cells (early adherent and chemoresistant) after their isolation. Then, the levels of EMT genes in the cancer cells incubated in conditioned media could be measured using qPCR analysis and compared with the levels of these genes in the cancer cells incubated in their normal growth media or in the conditioned media from unsorted cells.

3. It has been established that several soluble growth factors and cytokines induce activation of normal fibroblasts into CAFs such as, TGF- β , IL-6, VEGF, FGF, HGF, PDGF and SDF-1 (Kalluri and Zeisberg, 2006; Molloy *et al.*, 2009; Qian *et al.*, 2012). Therefore, it would be interesting to analyse the conditioned media of the isolated CSCs (early adherent and chemoresistant) to identify the specific proteins secreted from these cells which initiate activation of normal oral fibroblasts into CAFs. This could be conducted by analysing the collected conditioned media from the isolated CSCs using enzyme-linked immunosorbent assays (ELISA). Many reports suggest that TGF- β is the key factor in activation of normal fibroblasts to CAFs. Therefore, we could detect TGF- β in the conditioned media using ELISA and confirm its effect by using a specific inhibitor to block its effects. Similarly, we could use the same method to identify other specific molecules in cell secretion using ELISA or western blotting and use specific inhibitor to block their effects for confirmation of their role.
4. Our findings showed that isolated CSCs secreted high levels of EVs compare to unsorted cells. Therefore, it would be interesting to investigate if there is a role for secreted EVs in conditioned medium of isolated CSCs on activation of NOFs and what is their cargo. This could be performed through firstly isolation of the EVs from the conditioned medium of both isolated cells (early adherent and/ or chemoresistant) and unsorted cells using ultracentrifugation and then add them back to the fibroblast activation assay (in SFM) to confirm is it is the EVs having the effect. The proteins from the EVs could also be extracted and either the whole protein profile examined using proteomics or specific proteins levels investigated using western blotting. Nucleic acids could be analysed using sequencing or again specific micro RNAs could be analysed using qPCR.
5. It would be valuable to increase the number of oral squamous cell carcinoma patient cases in the studies of the immunohistochemical expression of CSC and stromal markers in order to allow for further correlations of their expression with different clinicopathological conditions. Furthermore, it is worthwhile to investigate the degree of significance of the correlation between CSC and activated stroma in oral dysplasia and in various stages of differentiation of oral squamous cell carcinoma (from well to poor differentiated). As a consequence,

it would allow for future developing of new diagnostic and prognostic biomarkers for oral squamous cell carcinoma.

List of presentations

1. Isolation and characterisation of cancer stem cells from oral cancer cell lines. *Sheffield Cancer Research Day*, 8th March 2017, Sheffield, England.
2. Isolation and characterisation of cancer stem cells from oral squamous cell carcinoma cell lines. *International Society for Stem Cells Research (ISSCR) Annual Conference*, 14-17th June 2017, Boston, USA.
3. Isolation and characterisation of oral cancer stem cells. *British Society for Oral and Dental Research (BSODR)*, 6-8th September 2017, Plymouth, England.
4. Cancer stem cells drive tumourgenesis through activation of stromal fibroblasts. *International Association of Dental Research (IADR) Annual Conference*, 24-28th July 2018, London, England.

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